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(54) Title: IMPROVED INTERLEUKIN-6 RECEPTOR ANTAGONIST

(57) Abstract

This invention provides novel improved interleukin-6 (IL-6) receptor antagonists that are human IL-6 muteins having five specific mutations, and receptor-binding fragments thereof. This invention also provides pharmaceutical compositions comprising these IL-6 receptor antagonists, and methods for treating IL-6 related diseases such as sepsis and multiple myeloma by administering such pharmaceutical compositions.

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IMPROVED INTERLEUKIN-6 RECEPTOR ANTAGONIST

Field of the Invention

This invention is in the field of immunology and control of host defense mechanisms. More specifically, this invention relates to the discovery of a class of interleukin-6 muteins (or variants) which interfere with the interaction between interleukin-6 and its two receptor proteins. This invention also relates to the use of such muteins to control and prevent interleukin-6 related diseases.

Background of the Invention

Interleukin-6 (IL-6) is a multi-functional cytokine playing a central role in host defense mechanisms. Heinrich et al., *Biochem. J., 265,* 621 (1990); Van Snick, *J. Annu. Rev. Immunol., 8,* 253 (1990); and Hirano et al., *Immunol. Today, 11,* 443 (1990). However, in a variety of human inflammatory, autoimmune, and neoplastic diseases, abnormal IL-6 production is observed and has been suggested to play a role in the pathogenesis of those diseases. Hirano et al., *supra*; Sehgal, *Proc. Soc. Exp. Biol. Med., 195,* 183 (1990); Grau, *Eur. Cytokine Net, 1,* 203 (1990); Bauer et al., *Ann. Hematol., 62,* 203 (1991); Campbell et al., *J. Clin. Invest., 7,* 739, (1991); and Roodman et al., *J. Clin. Invest., 89,* 46 (1992). Inhibitors of IL-6 bioactivity might thus be useful to study its role in disease and could have broad therapeutic applications.

IL-6 overproduction is involved in sepsis (Starnes, Jr. et al., *J. Immunol., 145,* 4185 (1990)), and is also implicated in multiple myeloma disease, or plasma cell leukemia (Klein et al., *Blood, 78,* 1198 (1991)). Other diseases include bone resorption (osteoporosis) (Roodman et al., *J. Clin. Invest., 89,* 46 (1992); Jilka et al., *Science, 257,* 88-91 (1992)), cachexia (Strassman et al., *J. Clin. Invest., 89,* 1681 (1992)), psoriasis, mesangial proliferative glomerulonephritis, renal cell carcinoma, Kaposi's sarcoma, AIDS-related lymphoma (Emilie et al., *Blood, 84*:2472-79 (1994)), rheumatoid arthritis, hypergammaglobulinemia (Grau et al., *J. Exp. Med. 172,* 1505

(1990)), Castleman's disease, IgM gammopathy, cardiac myxoma and autoimmune insulin-dependent diabetes mellitus (Campbell et al., *J. Clin. Invest., 87,* 739 (1991)).

IL-6 is a member of the long-chain family of cytokines and has an anti-parallel four alpha-helical bundle topology similar to growth hormone, granulocyte-colony stimulating factor and leukemia inhibitory factor. Sprang and Bazan, *Curr. Opin. Structural Biol., 3*:815 (1993); Hill et al., *Proc. Nat'l. Acad. Sci. USA, 90*:5167 (1993); Robinson et al., *Cell, 77*:1101 (1994). IL-6 functions through interaction with at least two specific receptors on the surface of target cells. Taga et al., *J. Exp. Med., 166,* 967 (1987); and Coulie et al., *Eur. J. Immunol., 17,* 1435 (1987). The cDNAs for these two receptor chains have been cloned, and they code for two transmembrane glycoproteins: the 80 kDa IL-6 receptor ("IL-6R") and a 130 kDa glycoprotein called "gp130". Yamasaki et al., *Science, 241,* 825 (1988); and Hibi et al., *Cell, 63,* 1149 (1990). IL-6 interacts with these glycoproteins following a unique mechanism. First, IL-6R binds to IL-6 with low affinity (Kd = about 1 nM) without triggering a signal. Taga et al., *Cell, 58,* 573 (1989). The IL-6/IL-6R complex subsequently associates with gp130 and induces disulfide-linked homodimerization and tyrosine phosphorylation of gp130, which results in signal transduction. Hibi et al., *supra*; and Taga et al., *supra*. Neither IL-6 nor IL-6R can bind independently to gp130. Gp130 stabilizes the IL-6/IL-6R complex on the membrane, resulting in high affinity binding of IL-6 (Kd = about 10 pM). Hibi et al., *supra*. Gp130 is also a low affinity receptor for oncostatin M and an affinity converter for the leukemia inhibitory factor (LIF) receptor (Gearing et al., *Science, 255,* 1434 (1992)). Gp130 is also part of the receptor complex of ciliary neurotrophic factor, IL-11 and the recently described cytokine cardiotrophin-1. Taga et al., *Curr. Opin. Immunol., 7*:17-23 (1995); Pennica et al., *Proc. Nat'l Acad. Sci. USA, 92*:1142-1146 (1995); Pennica et al., *J. Biol. Chem., 270*:10915-22 (1995).

Mature human (h) IL-6 is a 185 amino acid polypeptide containing two disulfide bonds (Cys_{45} to Cys_{51} and Cys_{74} to Cys_{84}. Clogston et al., *Arch. Biochem. Biophys., 272,* 144 (1989). The first 28 residues can be deleted without affecting bioactivity. Brakenhoff et al., *J. Immunol., 143,* 1175 (1989). Bioactivity of hIL-6

appears to be conformation dependent. Large internal deletions disrupt the overall structure of the molecule and completely abolish activity. Snouwaert et al., *J. Immunol., 146,* 585 (1991); and Fontaine et al., *Gene, 104,* 227 (1991). Maintenance of the second (but not the first) disulfide bond is critical, especially in bioassays involving human cell lines. Snouwaert et al., *J. Biol. Chem., 266,* 23097 (1991). Regions critical to activity comprise residues Ile_{30} to Asp_{35} (see Brakenhoff et al., *supra*; Fontaine et al., *supra*; and Arcone et al., *FEBS Letters, 288,* 197 (1991), reporting deletion of residues Leu_{39}-Glu_{42} without affecting activity), Ala_{154} to Thr_{164} (see Ida et al., *Biochem. Biophys. Res. Commun., 165,* 728 (1991); and Brakenhoff et al., *J. Biol., Chem., 269*:86 (1994)); and Nishimura et al., *FEBS Letters, 281,* 167 (1991)), and Arg_{183} to Met_{185} (see Krüttgen et al., *FEBS Letters, 262,* 323 (1990); Brakenhoff et al., *J. Immunol., 145,* 561 (1990); and Krüttgen et al., *FEBS Letters, 273,* 95 (1990)). Substitution analysis of individual residues have implicated Leu_{159}, Met_{162} and Leu_{166} to be important both for activity and binding to IL-6R (see Nishimura et al., *supra*. A positive charge and α-helical C-terminal structure were found to be essential for activity. Lütticken et al., *FEBS Letters, 282,* 265, (1991). Human-mouse chimeric proteins of IL-6 in which residues Lys_{42}-Ala_{57} or Cys_{51}-Glu_{56} of human IL-6 (hIL-6) were replaced with the corresponding residues of mouse IL-6 showed a strongly reduced capacity to trigger the binding of the IL-6/IL-6R complex to gp130. Ehlers et al., *J. Immunol., 153*:1744-1753 (1994); Ehlers et al., *J. Biol. Chem., 270*:8158-8163 (1995).

One method for neutralization of IL-6 activity is the use of antibodies to IL-6. Neutralizing monoclonal antibodies (mAbs) to IL-6 can be divided in two groups, based on the recognition of two distinct epitopes on the IL-6 molecule, designated Site I and Site II. Site I is a conformational epitope composed of both amino terminal and carboxy terminal portions of the IL-6 molecule: the amino terminal portion includes amino acids Ile_{30}-Asp_{35}; while the carboxy terminal portion includes critical amino acids Arg_{183}-Met_{185}. Site II includes critical amino acids Ala_{154}-Thr_{163}. Brakenhoff et al., *supra*, (1990).

Another way to neutralize IL-6 activity is to inhibit the ligand-receptor interactions with specific receptor-antagonists. The feasibility of this general type of approach was recently demonstrated with a naturally occurring receptor antagonist for interleukin-1. Hannum et al., *Nature, 343,* 336-340 (1990). However, no natural receptor-antagonist has been identified for IL-6 so far. Brakenhoff et al., Published PCT Application No. WO 94/09138, published April 28, 1994, discloses a class of hIL-6 muteins, preferably with one or more mutations in the region comprising amino acids 154-163, that act as IL-6 receptor antagonists.

However, there continues to exist a need for improved IL-6 receptor antagonists with greater antagonistic activity. Such antagonists will ideally be even more useful than the currently available antagonists for the treatment of IL-6 related diseases.

Summary of the Invention

The invention relates to novel hIL-6 receptor antagonists that function by binding to the IL-6 receptor (IL-6R) to form an hIL-6 variant/IL-6R complex that fails to interact with gp130. Specifically, the invention provides a human interleukin-6 (hIL-6) receptor antagonist comprising an modified hIL-6 protein (mutein) or a IL6-R receptor-binding fragment thereof comprising the following mutations: the amino acid at the position corresponding to residue 58 of mature hIL-6 protein is alanine, the amino acid at the position corresponding to residue 160 of mature hIL-6 protein is glutamic acid, the amino acid at the position corresponding to residue 163 of mature hIL-6 protein is proline, the amino acid at the position corresponding to residue 171 of mature hIL-6 protein is leucine, and the amino acid at the position corresponding to residue 177 of mature hIL-6 protein is arginine.

The invention is based on the discovery that a hIL-6 mutein (or variant) having these five mutations has unexpectedly improved antagonistic activity in *in vivo* assays of IL-6 activity. The hIL-6 mutein containing the combination of these five mutations has synergistic activity compared to other single-substitution mutants. It is contemplated that similar conservative substitutions may be made at these positions

without a significant loss of antagonistic activity. It is also contemplated that the substitutions may be made to fragments of IL-6 protein that retain IL-6R receptor-binding activity. For example, the first 28 amino-terminal amino acids of hIL-6 may be deleted without affecting its receptor-binding activity. An internal deletion of Leu_{39}-Glu_{42} may also be made without affecting receptor-binding activity.

The invention also provides pharmaceutical compositions for the treatment of IL-6 related diseases, i.e., diseases complicated by the production of IL-6 or wherein the overproduction of IL-6 is involved. These diseases include sepsis, multiple myeloma, plasma cell leukemia, osteoporosis, cachexia, psoriasis, mesangial proliferative glomerulonephritis, renal cell carcinoma, Kaposi's sarcoma, AIDS-related lymphoma, rheumatoid arthritis, hypergammaglobulinemia, Castleman's disease, IgM gammopathy, cardiac myxoma and autoimmune insulin-dependent diabetes. Such pharmaceutical compositions comprise a therapeutically effective amount of the hIL-6 receptor antagonist and a pharmaceutically acceptable carrier. Such carriers include aqueous solutions of isotonic or buffered saline, dextrose solutions and the like. A preferred pharmaceutical composition would be in unit dosage form and suitable for injection, preferably intravenously or intramuscularly.

The invention further provides a method for treating IL-6 related diseases comprising administering to a patient in need of such treatment a pharmaceutical composition comprising an amount of hIL-6 receptor antagonist effective for treating the IL-6 related disease. In preferred embodiments, the IL-6 related disease is sepsis or multiple myeloma.

Brief Description of the Drawings

Figure 1 shows a Coomassie blue-stained SDS-polyacrylamide gel of preparations of various hIL-6 mutants. The lanes contain: (1) rhIL-6 HGF7; (2) rhIL-6 $T_{163}P$; (3) rhIL-6 $Q_{160}E$, $T_{163}P$. The arrow denotes the migration position of mature rhIL-6.

Figures 2A-2C show the dose response curve of wild type hIL-6 and two hIL-6 mutants in various assays with human cell lines. Figure 2A shows the amount of IgG1 synthesis by CESS cells; Figure 2B shows the amount of C1 esterase inhibitor production by HepG2 cells; and Figure 2C shows the amount of tritiated thymidine incorporated by human myeloma cell line XG-1.

Figures 3A-3B show the inhibition of recombinant human IL-6 $Q_{160}E$, $T_{163}P$ on wild type hIL-6 activity. Figure 3A shows such inhibition in the CESS assay; and Figure 3B shows such inhibition in the HepG2 assay.

Figure 4 shows the amount of C1 esterase inhibitor production by HepG2 cells in the presence of wild type hIL-6 with and without hIL-6 $Q_{160}E$, $T_{163}P$.

Figure 5 shows the amount of C1 esterase inhibitor production by HepG2 cells in the presence of media, wild type hIL-6 (5 ng/ml) or gamma interferon (1 ng/ml) with and without hIL-6 $Q_{160}E$, $T_{163}P$.

Figure 6 compares the inhibition of IL-6 binding to IL-6 receptor-bearing cells (NIH-3T3 fibroblasts transfected with an expression vector encoding IL-6R) with rhIL-6 HGF7 and hIL-6 $Q_{160}E$, $T_{163}P$.

Figure 7 shows the activity of wild type hIL-6 and hIL-6-$L_{58}A/Q_{160}E/T_{163}P/F_{171}L/S_{177}R$ in the XG-1 bioassay.

Figure 8 shows the antagonistic effect of adding hIL-6-$L_{58}A/Q_{160}E/T_{163}P/F_{171}L/S_{177}R$ to rhIL-6 in the XG-1 bioassay.

Figure 9 shows the activity of wild type hIL-6 and hIL-6-$L_{58}A/Q_{160}E/T_{163}P/F_{171}L/S_{177}R$ in the murine B9 bioassay.

Figure 10 shows the relative ability of wild type hIL-6 and hIL-6-$L_{58}A/Q_{160}E/T_{163}P/F_{171}L/S_{177}R$ to inhibit binding of IL-6 to IL-6Rα.

Detailed Description of the Invention

The invention described herein draws on previously published work and pending patent applications. By way of example, such work consists of scientific papers, patents or pending patent applications. All of these publications and applications, cited previously or below are hereby incorporated by reference. Although any similar or equivalent methods and materials may be employed in the practice or testing of the present invention, the preferred methods and materials are now described.

Definitions:

The term "interleukin 6" or "IL-6", as used herein refers to IL-6 and to fragments, deletions, additions, substitutions, mutations and modifications thereof which retain the biological characteristics of the natural IL-6. Unless otherwise specified, the term refers to human IL-6.

The term "IL-6 related diseases" as used herein, refers to diseases associated with IL-6 overproduction including sepsis, multiple myeloma disease (plasma cell leukemia), bone resorption (osteoporosis), cachexia, psoriasis, mesangial proliferative glomerulonephritis, renal cell carcinoma, Kaposi's sarcoma, AIDS-related lymphoma, rheumatoid arthritis, hypergammaglobulinemia, Castleman's disease, IgM gammopathy, cardiac myxoma and autoimmune diabetes mellitus.

The term "IL-6 receptor antagonist", as used herein refers to molecules that interfere with the normal functioning of IL-6, as determined by specific inhibition of the wild type IL-6 molecule in in vitro bioassays, such as: (1) inhibition of IL-6 induction of production of acute phase proteins; (2) inhibition of IL-6 induction of myeloma or plasmacytoma growth; and (3) inhibition of IL-6 induction of immunoglobulin synthesis by human B cells. When the particular IL-6 receptor antagonist is a polypeptide of determined sequence, this invention also contemplates the term to include fragments, deletions, additions, substitutions, mutations and

modifications thereof which retain the biological characteristics of the determined polypeptide.

A "mutation" in a protein alters its primary structure due to changes in the nucleotide sequence of the DNA which encodes it. These mutations include allelic variants. A "modified" protein differs from the unmodified protein as a result of post-translational events which change the glycosylation or lapidation pattern, or the primary, secondary, or tertiary structure of the protein. Changes in the primary structure of a protein can also result from deletions, additions, or substitutions. A "deletion" is defined as a polypeptide in which one or more internal amino acid residues are absent. An "addition" is defined as a polypeptide which has one or more additional internal amino acid residues as compared to the wild type. A "substitution" results from the replacement of one or more amino acid residues by other residues. A protein "fragment" is a polypeptide consisting of a primary amino acid sequence which is identical to a portion of the primary sequence of the protein to which the polypeptide is related.

Preferred "substitutions" are those which are conservative, i.e., wherein a residue is replaced by another of the same general type. As is well understood, naturally-occurring amino acids can be subclassified as acidic, basic, neutral and polar, or neutral and nonpolar. Furthermore, three of the encoded amino acids are aromatic. It is generally preferred that encoded peptides differing from the determined IL-6 receptor antagonist contain substituted codons for amino acids which are from the same group as that of the amino acid replaced. Thus, in general, the basic amino acids Lys, Arg, and His are interchangeable; the acidic amino acids Asp and Glu are interchangeable; the neutral polar amino acids Ser, Thr, Cys, Gln, and Asn are interchangeable; the nonpolar aliphatic amino acids Gly, Ala, Val, Ile, and Leu are conservative with respect to each other (but because of size, Gly and Ala are more closely related and Val, Ile and Leu are more closely related), and the aromatic amino acids Phe, Trp and Tyr are interchangeable.

It should further be noted that if IL-6 receptor antagonist polypeptides are made synthetically, substitutions by amino acids which are not naturally encoded by DNA

may also be made. For example, alternative residues include the omega amino acids of the formula $NH_2(CH_2)_nCOOH$ wherein n is 2-6. These are neutral, nonpolar amino acids, as are sarcosine, t-butyl alanine, t-butyl glycine, N-methyl isoleucine, and norleucine. Phenylglycine may substitute for Trp, Tyr or Phe; citrulline and methionine sulfoxide are neutral polar, cyclohexylalanine is neutral nonpolar, cysteic acid is acidic, and ornithine is basic. Proline may be substituted with hydroxyproline and retain the conformation conferring properties.

The "biological characteristics" of a protein refers to the structural or biochemical function of the protein in the biological process of the organism in which it participates. Examples of biological characteristics of IL-6 receptor antagonists include: (1) inhibition of IgG_1 synthesis by CESS cells induced by wild type IL-6; (2) induction of C1 esterase inhibitor synthesis by HepG2 cells induced by wild type IL-6; (3) ability to bind to the IL-6 receptor without activity on IL-6-responsive cells; (4) competition with wild type IL-6 for binding to the IL-6 receptor; and (5) inhibition of biological activity of wild type IL-6 on target cells.

As used herein, "Site I" refers to a region of the hIL-6 molecule that plays a role in the binding of IL-6 to its receptor (IL-6R). The region includes sites at both the amino terminal and carboxy terminal portions of the IL-6 molecule: the amino terminal portion includes amino acids Ile_{30}-Asp_{35}; while the carboxy terminal portion includes critical amino acids Arg_{183}-Met_{185}. A portion of this region is recognized by the monoclonal antibody mAb CLB.IL-6/8 (see Brakenhoff et al., *J. Immunol., 145,* 561 (1990)). "Site II" plays a role in the interaction of the IL-6/IL-6R complex with gp130, and includes critical amino acids Ala_{154}-Thr_{163}. This conformational epitope on the IL-6 molecule is recognized by the monoclonal antibody mAb CLB.IL-6/16 (see Brakenhoff et al., *J. Immunol., 145,* 561 (1990)).

Preparation of IL-6 Receptor Antagonists

The sequence of the mature human IL-6 protein, deduced from the cDNA sequence, is known in the art [see Figure 1 of Van Snick, *supra*] and for convenience is reproduced herein in SEQ ID NO: 5.

IL-6 receptor antagonists may be produced synthetically by the method of Merrifield et al. IL-6 receptor antagonists may be produced recombinantly as shown in U.S. Patent No. 4,966,852. For example, the cDNA for the protein can be incorporated into a plasmid for expression in prokaryotes or eukaryotes. U.S. Patent No. 4,847,201, which is hereby incorporated by reference in its entirety, provides details for transforming microorganisms with specific DNA sequences and expressing them. There are many other references known to those of ordinary skill in the art which provide details on expression of proteins using microorganisms. Many of those are cited in U.S. Patent No. 4,847,201, such as Sambrook et al., *Molecular Cloning,* Cold Spring Harbor Press (2d ed. 1989).

The following is an overview about transforming and expressing IL-6 receptor antagonists in microorganisms. IL-6 receptor antagonists DNA sequences may be incorporated into plasmids, such as pUNC13 or pBR3822, which are commercially available from companies such as Boehringer-Mannheim. Once the IL-6 receptor antagonist DNA is inserted into a vector, it can be cloned into a suitable host. The DNA can be amplified by techniques such as those shown in U.S. Patent No. 4,683,202 to Mullis and U.S. Patent No. 4,683,195 to Mullis et al. After the expression vector is transformed into a host such as *E. coli* the bacteria may be fermented and the protein expressed. Bacteria are preferred prokaryotic microorganisms and *E. coli* is especially preferred. A preferred microorganism useful in the present invention is *E. coli* K-12, strain MM294 deposited with the American Type Culture Collection, 12301 Parklawn Drive, Rockville, Maryland, 20852, United States of America (hereinafter referred to as "ATCC"), on February 14, 1984, under the provisions of the Budapest Treaty, Accession Number 39607. Alternatively, IL-6 receptor antagonists may be introduced into mammalian cells. These mammalian cells

may include CHO, COS, C127, Hep G2, SK Hep, baculovirus, and infected insect cells (see also U.S. Patent No. 4,847,201, referred to above). See also Pedersen et al., *J. Biol. Chem., 265,* 16786-16793 (1990).

Some specific details about the production of a recombinant protein typically involve the following:

Suitable Hosts, Control Systems and Methods

First, a DNA encoding the mature protein (used here to include all muteins); the preprotein; or a fusion of the IL-6 receptor antagonist protein to an additional sequence which does not destroy its activity or to additional sequence cleaved under controlled conditions (such as treatment with peptidase) to give an active protein, is obtained. If the sequence is uninterrupted by introns it is suitable for expression in any host. If there are introns, expression is obtainable in mammalian or other eucaryotic systems capable of processing them. This sequence should be in excisable and recoverable form. The excised or recovered coding sequence is then placed in operable linkage with suitable control sequences in a replicable expression vector. The vector is used to transform a suitable host and the transformed host cultured under favorable conditions to effect the production of the recombinant IL-6 receptor antagonists.

Genomic or cDNA fragments are obtained and used directly in appropriate hosts. The constructions for expression vectors operable in a variety of hosts are made using appropriate replications and control sequences, as set forth below. Suitable restriction sites can, if not normally available, be added to the ends of the coding sequence so as to provide an excisable gene to insert into these vectors.

The control sequences, expression vectors, and transformation methods are dependent on the type of host cell used to express the gene. Generally, procaryotic, yeast, or mammalian cells are presently useful as hosts. Host systems which are capable of proper post-translational processing are preferred. Accordingly, although procaryotic hosts are in general the most efficient and convenient for the production of recombinant proteins, eucaryotic cells, and, in particular, mammalian cells are

preferred for their processing capacity, for example, the ability to form the proper glycosylation patterns. In addition, there is more assurance that the native signal sequence will be recognized by the mammalian host cell, thus making secretion possible, and purification thereby easier.

Control Sequences and Corresponding Hosts

Procaryotes most frequently are represented by various strains of *E. coli*. However, other microbial strains may also be used, such as bacilli, for example *Bacillus subtilis*, various species of Pseudomonas, or other bacterial strains. In such procaryotic systems, plasmid vectors which contain replication sites and control sequences derived from a species compatible with the host are used. For example, *E. coli* is typically transformed using derivatives of pBR322, a plasmid derived from an *E. coli* species by Bolivar et al., *Gene, 2,* 95 (1977). pBR322 contains genes for ampicillin and tetracycline resistance, and thus provides additional markers which can be either retained or destroyed in constructing the desired vector. Commonly used procaryotic control sequences are defined herein to include promoters for transcription initiation, optionally with an operator, along with ribosome binding site sequences, which include such commonly used promoters as the beta-lactamase (penicillinase) and lactose (lac) promoter systems (Chang et al., *Nature, 198,* 1056 (1977)); the tryptophan (trp) promoter system (Goeddel, et al., *Nucleic Acids Res., 8,* 4057 (1980)); the T7 promoter (Studier et al., *Meth. Enzymol., 185,* 60 (1990)); and the λ derived P_L promoter and N-gene ribosome binding site (Shimatake et al., *Nature, 292,* 128 (1981)), which has been made useful as a portable control cassette, as set forth in U.S. Patent No. 4,711,845, issued December 8, 1987. However, any available promoter system compatible with procaryotes can be used.

In addition to bacteria, eucaryotic microbes, such as yeast, may also be used as hosts. Laboratory strains of *Saccharomyces cerevisiae*, Baker's yeast, are most used although a number of other strains are commonly available. Examples of plasmid vectors suitable for yeast expression are shown in Broach, *Meth. Enz., 101,* 307 (1983); Stinchcomb et al., *Nature, 282,* 39 (1979); and Tschempe et al., *Gene, 10,*

157 (1980) and Clarke et al., *Meth. Enz., 101,* 300 (1983). Control sequences for yeast vectors include promoters for the synthesis of glycolytic enzymes (Hess et al., *J. Adv. Enzyme Reg., 7,* 149 (1968); Holland et al., *Biochemistry, 17,* 4900 (1978)). Additional promoters known in the art include the promoter for 3- phosphoglycerate kinase (Hitzeman et al., *J. Biol. Chem., 255,* 2073 (1980)), and those for other glycolytic enzymes, such as glyceraldehyde-3-phosphate dehydrogenase, hexokinase, pyruvate decarboxylase, phosphofructokinase, glucose-6-phosphate isomerase, 3-phosphoglycerate mutase, pyruvate kinase, triosephosphate isomerase, phosphoglucose isomerase, and glucokinase. Other promoters, which have the additional advantage of transcription controlled by growth conditions, are the promoter regions for alcohol dehydrogenase 2, isocytochrome C, acid phosphatase, degradative enzymes associated with nitrogen metabolism, and enzymes responsible for maltose and galactose utilization (Holland, *supra*). It is also believed that terminator sequences are desirable at the 3' end of the coding sequences. Such terminators are found in the 3' untranslated region following the coding sequences in yeast-derived genes. Many of the vectors illustrated contain control sequences derived from the enolase gene containing plasmid peno46 (Holland et al., *J. Biol. Chem., 256,* 1385 (1981)) or the LEU2 gene obtained from YEpl3 (Broach et al., *Gene, 8,* 121 (1978)), however, any vector containing a yeast compatible promoter, origin of replication and other control sequences is suitable.

It is also, of course, possible to express genes encoding polypeptides in eucaryotic host cell cultures derived from multicellular organisms. See, for example, *Tissue Culture*, 1973, Cruz and Patterson, eds., Academic Press. Useful host cell lines include murine myelomas N51; VERO, HeLa cells, Chinese hamster ovary (CHO) cells, COS, C127, Hep G2, SK Hep, baculovirus, and infected insect cells. Expression vectors for such cells ordinarily include promoters and control sequences compatible with mammalian cells such as, for example, the commonly used early and later promoters from Simian Virus 40 (SV40) (Fiers et al., *Nature, 273,* 113 (1978)), or other viral promoters such as those derived from polyoma, Adenovirus 2, bovine papilloma virus, or avian sarcoma viruses, or immunoglobulin promoters and heat

shock promoters. General aspects of mammalian cell host system transformations have been described by Axel, U.S. Patent No. 4,399,216, issued August 16, 1983. It now appears also that "enhancer" regions are important in optimizing expression; these are, generally, sequences found upstream of the promoter region. Origins of replication may be obtained, if needed, from viral sources. However, integration into the chromosome is a common mechanism for DNA replication in eucaryotes. Plant cells are also now available as hosts, and control sequences compatible with plant cells such as the nopaline synthase promoter and polyadenylation signal sequences (Depicker et al., *J. Mol. Appl. Gen., 1,* 561 (1982)) are available. Methods and vectors for transformation of plant cells have been disclosed in PCT Publication No. WO 85/04899, published November 7, 1985.

Host strains useful in cloning and expression herein are as follows:

For cloning and sequencing, and for expression of construction under control of most bacterial promoters, *E. coli* strain MM294 obtained from *E. coli* Genetic Stock Center GCSC #6135. For expression under control of the P_LN_{RBS} promoter, *E. coli* strain K12 MC1000 lambda lysogen, N_7N_{53}cI857 SusP80, a strain deposited with the ATCC on December 2, 1983 under the provisions of the Budapest Treaty, Accession Number 39531, may be used. *E. coli* DG116, which was deposited with the ATCC on April 7, 1987, under the provisions of the Budapest Treaty, Accession No. 53606, may also be used.

For M13 phage recombinants, *E. coli* strains susceptible to phage infection, such as *E. coli* K12 strain DG98, can be employed. The DG98 strain has been deposited with the ATCC on July 13, 1984, under the provisions of the Budapest Treaty, Accession No. 39768.

Mammalian expression can be accomplished in COS-A2 cells, COS-7, CV-1, murine myelomas N51, VERO, HeLa cells, Chinese hamster ovary (CHO) cells, COS, C127, Hep G2, SK Hep, baculovirus, and infected insect cells. Insect cell-based expression can be in *Spodoptera frugiperda*.

Transformations

Depending on the host cell used, transformation is done using standard techniques appropriate to such cells. The calcium treatment employing calcium chloride, as described by Cohen, *Proc. Nat'l. Acad. Sci. (USA), 69,* 2110 (1972), is used for procaryotes or other cells which contain substantial cell wall barriers. Infection with *Agrobacterium tumefaciens* (Shaw et al., *Gene, 23,* 315 (1983)) is used for certain plant cells. For mammalian cells without such cell walls, the calcium phosphate precipitation method of Graham et al., *Virology, 52,* 546 (1987) is preferred. Transformations into yeast are carried out according to the method of Van Solingen et al., *J. Bact., 130,* 946 (1977) and Hsiao et al., *Proc. Nat'l. Acad. Sci. (USA), 76,* 3829 (1979).

Vector Construction

Construction of suitable vectors containing the desired coding and control sequences employs standard ligation and restriction techniques which are well understood in the art. Isolated plasmids, DNA sequences, or synthesized oligonucleotides are cleaved, tailored, and religated in the form desired.

Site specific DNA cleavage is performed by treating with the suitable restriction enzyme (or enzymes) under conditions which are generally understood in the art, and the particulars of which are specified by the manufacturer of these commercially available restriction enzymes. See, e.g., New England Biolabs, Product Catalog. In general, about 1 μg of plasmid or DNA sequence is cleaved by 1 unit of enzyme in about 20 μl of buffer solution; in the examples herein, typically, an excess of restriction enzyme is used to insure complete digestion of the DNA substrate. Incubation times of about 1 hour to 2 hours at about 37°C are workable, although variations can be tolerated. After each incubation, protein is removed by extraction with phenol/chloroform, and may be followed by ether extraction, and the nucleic acid recovered from aqueous fractions by precipitation with ethanol. If desired, size separation of the cleaved fragments may be performed by polyacrylamide gel or

agarose gel electrophoresis using standard techniques. A general description of size separations is found in *Methods of Enzymology, 65,* 499-560 (1980).

Synthetic oligonucleotides may be prepared by the triester method of Matteucci et al., *J. Am. Chem. Soc., 103,* 3185-3191 (1981), or using automated synthesis methods. Kinasing of single strands prior to annealing or for labelling is achieved using an excess, e.g., approximately 10 units of polynucleotide kinase to 1 nmole substrate in the presence of 50 mM Tris, pH 7.6, 10 mM $MgCl_2$, 5 mM DTT, 1-2 mM ATP. If kinasing is for labelling of probe, the ATP will contain high specific activity γ-^{32}P.

Ligations are performed in 15-30 µl volumes under the following standard conditions and temperatures: 20 mM Tris-Cl pH 7.5, 10 mM $MgCl_2$, 10 mM DTT, 33 µg/ml bovine serum albumin (BSA), 10 mM-50 mM NaCl, and either 40 µM ATP, 0.01-0.02 (Weiss) units T4 DNA ligase at 0°C (for "sticky end" ligation) or 1 mM ATP, 0.3-0.6 (Weiss) units T4 DNA ligase at 14°C (for "blunt end" ligation). Intermolecular "sticky end" ligations are usually performed at 33-100 µg/ml total DNA concentrations (5-100 nM total end concentration). Intermolecular blunt end ligations (usually employing a 10-30 fold molar excess of linkers) are performed at 1 µM total ends concentration.

In the vector construction employing "vector fragments", the vector fragment is commonly treated with bacterial alkaline phosphatase (BAP) in order to remove the 5' phosphate and prevent religation of the vector. BAP digestions are conducted at pH 8 in approximately 150 mM Tris, in the presence of Na^{2+} and Mg^{2+} using about 1 unit of BAP per µg of vector at 60°C for about 1 hour. In order to recover the nucleic acid fragments, the preparation is extracted with phenol/chloroform and ethanol precipitated. Alternatively, religation can be prevented in vectors which have been double digested by additional restriction enzyme digestion of the unwanted fragments.

Modification of DNA Sequences

For portions of vectors derived from cDNA or genomic DNA which require sequence modifications, site specific primer directed mutagenesis is used. This technique is now standard in the art, and is conducted using a primer synthetic oligonucleotide complementary to a single stranded phage DNA to be mutagenized except for limited mismatching, representing the desired mutation. Briefly, the synthetic oligonucleotide is used as a primer to direct synthesis of a strand complementary to the phage, and the resulting double-stranded DNA is transformed into a phage-supporting host bacterium. Cultures of the transformed bacteria are plated in top agar, permitting plaque formation from single cells which harbor the phage.

Theoretically, 50% of the new plaques will contain the phage having, as a single strand, the mutated form: 50% will have the original sequence. The plaques are hybridized with kinased synthetic primer at a temperature which permits hybridization of an exact match, but at which the mismatches with the original strand are sufficient to prevent hybridization. Plaques which hybridize with the probe are then picked, cultured, and the DNA recovered.

Verification of Construction

Correct ligations for plasmid construction could be confirmed by first transforming *E. coli* strain MM294, or other suitable host, with the ligation mixture. Successful transformants are selected by ampicillin, tetracycline or other antibiotic resistance or using other markers depending on the mode of plasmid construction, as is understood in the art. Plasmids from the transformants are then prepared according to the method of Clewell et al., *Proc. Nat'l. Acad. Sci. (USA), 62,* 1159 (1969), optionally following chloramphenicol amplification (Clewell *J. Bacteriol, 110,* 667 (1972)). The isolated DNA is analyzed by restriction and/or sequenced by the dideoxy method of Sanger et al., *Proc. Nat'l. Acad. Sci. (USA), 74,* 5463 (1977) as further

described by Messing et al., *Nucleic Acids Res., 9,* 309 (1981), or by the method of Maxam et al., *Methods in Enzymology, 65,* 499 (1980).

Purification of IL-6 Receptor Antagonists

IL-6 receptor antagonists may be produced in bacteria, such as *E. coli*, and subsequently purified. Generally, the procedures shown in U.S. Patent Nos. 4,511,502; 4,620,948; 4,929,700; 4,530,787; 4,569,790; 4,572,798; and 4,748,234 can be employed. These patents are hereby incorporated by reference in their entireties. Typically, the heterologous protein (i.e., IL-6 receptor antagonist) is produced in a refractile body within the bacteria. To recover and purify the protein, the cells are lysed and the refractile bodies are centrifuged to separate them from the cellular debris (see U.S. Patent No. 4,748,234 for lowering the ionic strength of the medium to simplify the purification). Thereafter, the refractile bodies containing the IL-6 receptor antagonist are denatured, at least once (typically in a non-reducing environment), and the protein is oxidized and refolded in an appropriate buffer solution for an appropriate length of time. IL-6 receptor antagonists may be purified from the buffer solution by various chromatographic methods, such as those mentioned above for the mammalian cell derived IL-6 receptor antagonists. Preferably, IL-6 receptor antagonists are purified by affinity chromatography using anti-IL-6 monoclonal antibodies. Additionally, the methods shown in U.S. Patent No. 4,929,700 may be employed.

Formation and Administration

IL-6 receptor antagonists are administered at a concentration that is therapeutically effective to treat and prevent IL-6 related diseases, including sepsis and multiple myeloma. To accomplish this goal, IL-6 receptor antagonists are preferably administered intravenously. Methods to accomplish this administration are known to those of ordinary skill in the art.

Before administration to patients, formulants may be added to IL-6 receptor antagonists. A liquid formulation is preferred. For example, these formulants may include oils, polymers, vitamins, carbohydrates, amino acids, salts, buffers, albumin, surfactants, or bulking agents. Preferably carbohydrates include sugar or sugar alcohols such as mono, di, or polysaccharides, or water soluble glucans. The saccharides or glucans can include fructose, dextrose, lactose, glucose, mannose, sorbose, xylose, maltose, sucrose, dextran, pullulan, dextrin, alpha and beta cyclodextrin, soluble starch, hydroxethyl starch and carboxymethylcellulose, or mixtures thereof. Sucrose is most preferred. "Sugar alcohol" is defined as a C_4 to C_8 hydrocarbon having an -OH group and includes galactitol, inositol, mannitol, xylitol, sorbitol, glycerol, and arabitol. Mannitol is most preferred. These sugars or sugar alcohols mentioned above may be used individually or in combination. There is no fixed limit to amount used as long as the sugar or sugar alcohol is soluble in the aqueous preparation. Preferably,the sugar or sugar alcohol concentration is between 1.0 w/v% and 7.0 w/v%, more preferable between 2.0 and 6.0 w/v%. Preferably amino acids include levorotary (L) forms of carnitine, arginine, and betaine; however, other amino acids may be added. Preferred polymers include polyvinylpyrrolidone (PVP) with an average molecular weight between 2,000 and 3,000, or polyethylene glycol (PEG) with an average molecular weight between 3,000 and 5,000. It is also preferred to use a buffer in the composition to minimize pH changes in the solution before lyophilization or after reconstitution. Most any physiological buffer may be used, but citrate, phosphate, succinate, and glutamate buffers or mixtures thereof are preferred. Most preferred is a citrate buffer. Preferably, the concentration is from 0.01 to 0.3 molar. Surfactants that can be added to the formulation are shown in EP Nos. 270,799 and 268,110.

Additionally, IL-6 receptor antagonists can be chemically modified by covalent conjugation to a polymer to increase its circulating half-life, for example. Preferred polymers, and methods to attach them to peptides, are shown in U.S. Patent Nos. 4,766,106; 4,179,337; 4,495,285; and 4,609,546 which are all hereby incorporated by reference in their entireties. Preferred polymers are polyoxyethylated polyols and

polyethylene glycol (PEG). PEG is soluble in water at room temperature and has the general formula: $R(O-CH_2-CH_2)_nO-R$ where R can be hydrogen, or a protective group such as an alkyl or alkanol group. Preferably, the protective group has between 1 and 8 carbons, more preferably it is methyl. The symbol n is a positive integer, preferably between 1 and 1,000, more preferably between 2 and 500. The PEG has a preferred average molecular weight between 1000 and 40,000, more preferably between 2000 and 20,000, most preferably between 3,000 and 12,000. Preferably, PEG has at least one hydroxy group, more preferably it is a terminal hydroxy group. It is this hydroxy group which is preferably activated to react with a free amino group on the inhibitor. However, it will be understood that the type and amount of the reactive groups may be varied to achieve a covalently conjugated PEG/IL-6 receptor antagonist of the present invention.

Water soluble polyoxyethylated polyols are also useful in the present invention. They include polyoxyethylated sorbitol, polyoxyethylated glucose, polyoxyethylated glycerol (POG), etc. POG is preferred. One reason is because the glycerol backbone of polyoxyethylated glycerol is the same backbone occurring naturally in, for example, animals and humans in mono-, di-, triglycerides. Therefore, this branching would not necessarily be seen as a foreign agent in the body. The POG has a preferred molecular weight in the same range as PEG. The structure for POG is shown in Knauf et al., *J. Bio. Chem., 263,* 15064-15070 (1988) and a discussion of POG/IL-2 conjugates is found in U.S. Patent No. 4,766,106, both of which are hereby incorporated by reference in their entireties.

After the liquid pharmaceutical composition is prepared, it is preferably lyophilized to prevent degradation and to preserve sterility. Methods for lyophilizing liquid compositions are known to those of ordinary skill in the art. Just prior to use, the composition may be reconstituted with a sterile diluent (Ringer's solution, distilled water, or sterile saline, for example) which may include additional ingredients. Upon reconstitution, the composition is preferably administered to subjects using those methods that are known to those skilled in the art.

Administration to Affected Individuals

As stated above, IL-6 receptor antagonists are useful to treat human patients with IL-6 related diseases, including sepsis and multiple myeloma. Generally, sepsis patients are characterized by high fever (>38.5°C) or hypothermia (<35.5°C), low blood pressure, tachypnea (> than 20 breaths/minute), tachycardia (> than 100 beats/minute), leukocytosis (> 15,000 cells/mm^3) and thrombocytopenia (< than 100,000 platelets/mm^3) in association with bacteremia. IL-6 receptor antagonists are to be administered as soon as a patient is suspected of being septic; presenting themselves with a > 20% drop in fibrinogen or appearance of fibrin split products, a rise in the patient's temperature and the diagnosis of leukopenia and hypotension associated with sepsis. As also stated above, the preferred route is by intravenous administration. Generally, IL-6 receptor antagonists are given at a dose between 1 μg/kg and 20 mg/kg, more preferably between 20 μg/kg and 10 mg/kg, most preferably between 1 and 7 mg/kg. Preferably, it is given as a bolus dose, to increase circulating levels by 10-20 fold and for 4-6 hours after the bolus dose. Continuous infusion may also be used after the bolus dose. If so, IL-6 receptor antagonists may be infused at a dose between 5 and 20 μg/kg/minute, more preferably between 7 and 15 μg/kg/minute.

When used to treat sepsis, IL-6 receptor antagonists may be given in combination with other agents which would be effective to treat sepsis. For example, the following may be administered in combination with IL-6 receptor antagonists: antibiotics that can treat the underlying bacterial infection; monoclonal antibodies that are directed against bacterial cell wall components; receptors that can complex with cytokines that are involved in the sepsis pathway; antibodies to cell adhesion molecules such as LFA-1; and generally any agent or protein that can interact with cytokines or complement proteins in the sepsis pathway to reduce their effects and to attenuate sepsis or septic shock.

IL-6 receptor antagonists may also be administered in conjunction with other similar modulatory cytokines including LIF, oncostatin M, CNTF and IL-11.

Antibiotics that are useful in the present invention include those in the general category of: beta-lactam rings (penicillin), amino sugars in glycosidic linkage (amino glycosides), macrocyclic lactone rings (macrolides), polycyclic derivatives of napthacenecarboxamide (tetracyclines), nitrobenzene derivatives of dichloroacetic acid, peptides (bacitracin, gramicidin, and polymyxin), large rings with a conjugated double bond system (polyenes), sulfa drugs derived from sulfanilamide (sulfonamides), 5-nitro-2-furanyl groups (nitrofurans), quinolone carboxylic acids (nalidixic acid), and many others. Other antibiotics and more versions of the above specific antibiotics may be found in Encyclopedia of Chemical Technology, 3rd Edition, Kirk-Othymer (ed.), Vol. 2, pages 782-1036 (1978) and Vol. 3, pages 1-78, Zinsser, *MicroBiology*, 17th Ed., Joklik et al. (Eds.) 235-277 (1980), or Dorland's Illustrated Medical Dictionary, 27th Ed., W.B. Saunders Company (1988).

Monoclonal antibodies that may be administered along with IL-6 receptor antagonists include those found in PCT WO 88/03211, to Larrick et al., entitled Gram-Negative Bacterial Endotoxin Blocking Monoclonal Antibodies, and U.S. Serial No. 07/876,854, filed April 30, 1992, to Larrick et al. Both applications disclose specific monoclonal antibodies that are useful to treat sepsis and which bind to various antigens on the *E. coli* bacterial cell wall. A specifically preferred monoclonal antibody is that which is produced by hybridoma deposited with the ATCC on May 19, 1987, under the provisions of the Budapest Treaty, Accession No. HB9431.

Other agents which may be combined with IL-6 receptor antagonists include monoclonal antibodies directed to cytokines involved in the sepsis pathway, such as those monoclonal antibodies directed to IL-6 or M-CSF, see U.S. Serial No. 07/451,218, filed December 15, 1989 to Creasey et al. and monoclonal antibodies directed to TNF, see Cerami et al., U. S. Patent No. 4,603,106. Inhibitors of protein that cleave the mature TNF prohormone from the cell in which it was produced, see U.S. Serial No. 07/395,253, filed August 16, 1989, to Kriegler et al. Antagonists of IL-1, such as shown in U.S. Serial No. 07/517,276, filed May 1, 1990 to Haskill et al. Inhibitors of IL-6 cytokine expression such as inhibin, such as shown in U.S. Serial

No. 07/494,624, filed March 16, 1992, to Warren et al., and receptor based inhibitors of various cytokines such as IL-1. Antibodies to complement may also be employed.

Generally, IL-6 receptor antagonists may be useful for those diseases that occur due to the up-regulation of tissue factor brought on by TNF, IL-1 or other cytokines.

The present invention will now be illustrated by reference to the following examples which set forth particularly advantageous embodiments. However, it should be noted that these embodiments are illustrative and are not to be construed as restricting the invention in any way.

Example 1

Preparation of hIL-6 Variants
With Mutations In or Around the Region Gln153-Thr163

Materials and Methods

Antibodies and Cytokines: The production and purification of an IL-6 specific mAb has been described before in detail (see Brakenhoff et al. (1990)). The wild-type rIL-6 preparation used in this example as a standard is purified from *E. coli* carrying the HGF7 plasmid (see Brakenhoff et al., *J. Immunol., 139,* 4116 (1987)). HGF7 encodes an IL-6 fusion protein consisting of a 9 amino acid β-galactosidase derived leader followed by 4 glycines, an aspartic acid residue, and Arg_{17}-Met_{185} of mature IL-6. Purification of HGF7 has been described before in Brakenhoff et al. (1990). Specific activity of purified rhIL-6 HGF7 as determined in the B9 assay is approximately 10^9 U/mg. Recombinant IFN-γ was obtained from Genentech (San Francisco, California).

Bacterial Strains and Vectors: Construction of the expression vector pUK-IL-6 is described in Brakenhoff et al., 1989. Expression of vectors occurred in an *E. coli* DH5α (GIBCO BRL) host.

A. Preparation of an IL-6 Variant Library

Random mutagenesis of the hIL-6 region around Trp_{158} was performed to identify residues which might be important to the biological activity of hIL-6. The vector pUK-IL-6 was used for construction of the library of rhIL-6 mutants with random substitutions in residues Gln_{153}-Thr_{163}. Restriction fragments with the desired substitutions suitable for subcloning in pUK-IL-6, were obtained in two steps by overlap extension PCR (see Ho et al., *Gene, 77,* 51 (1989)). In the first PCR reactions, pUK-IL-6 was used as template. Fragment 1, running from the unique XbaI site in the hIL-6 coding region, to amino acid Thr_{163}, was generated by combining a 5' primer (A) with (SEQ ID NO: 1) (nucleotides (nts) 477-498 of hIL-6 coding region (see Brakenhoff et al., *supra,* 1987, and a 3' primer (B) with (SEQ ID NO: 2) (nts 537-570, corresponding to residues Gln_{153}-Thr_{163}). To obtain randomly distributed substitutions in primer B a similar approach was used as described by Derbyshire et al., *Gene, 46,* 145 (1986). Instead of contaminating each nucleotide reservoir with the three other monomers, the fifth channel of the oligo synthesizer (Applied Biosystems type 381A, Warrington, UK) was used during oligo synthesis: during each synthesis step both the channel containing 100 mM of the wild-type nucleotide and the fifth channel containing 1.25 mM of each of the four dNTP's were mixed in 1:1 ratio. With an oligo length of 34 this results in approximately 36% single, 36% multiple, and 28% no mutations per oligo. Fragment 2, running from Gln_{153} to the BanII site, was generated by using a 5' primer C complementary to primer B (SEQ ID NO: 3) (nts 538-571) synthesized in the same manner as primer B. This oligo was combined with a 3' primer D, (SEQ ID NO: 4) (nts 609-629). PCRs were carried out with Taq polymerase as specified by the manufacturer (Perkin Elmer Cetus) by using 10 ng of template DNA and 100 ng of each primer (annealing 2 minutes at 50°C, extension 2.5 minutes at 65°C, denaturation 1.5 minutes at 95°C; 30 cycles). After the first PCR reactions, fragments 1 and 2 were purified from low melting agarose and approximately 100 ng of each served as template in the second PCR reaction with primers A and D. After phenol/$CHCl_3$ extraction the second PCR product was digested

with XbaI and BanII, gel purified and subcloned in XbaI-BanII digested pUK-IL-6. Following transformation to *E. coli* DH5α approximately 1,000 colonies were obtained. DNA manipulation procedures were performed as described in Brakenhoff et al., 1989 and Brakenhoff et al., 1990. Nucleotide sequences of selected mutants (see below) were obtained with cDNA derived oligonucleotide primers on dsDNA by using the "Sequenase" kit (United States Biochemical Corporation, Cleveland, OH).

B. Preparation and Screening of hIL-6 Variants

Variants were subsequently selected for binding to a site I specific mAb (mAb CLB.IL-6/8) and loss of binding to a site II specific mAb (mAb CLB.IL-6/16) in ELISAs and the nucleotide sequence of plasmids encoding mutants with this phenotype was determined. 400 ampicillin resistant colonies were toothpicked in wells of 96-well flat bottom microtiterplates (NUNC) containing 100 µl LC amp medium (10 g of bactotryptone, 5 g of yeast extract, 8 g of NaCl, and 2 ml of Tris base per liter supplemented with 100 µg/ml final concentration of ampicillin). Following overnight culture at 37°C, bacteria were lysed by addition of lysozyme to 1 mg/ml and further incubation for 30 minutes at 37°C. One in 10 dilutions of the crude extracts in phosphate-buffered saline (PBS), 0.02% Tween-20, 0.2% gelatin were directly tested for reactivity in sandwich ELISAs with mAb CLB.IL-6/8 or 16 coated to the plastic and biotinylated polyclonal goat anti-rhIL-6 as detecting antibody. Bound polyclonal anti-rhIL-6 was detected with horseradish peroxidase conjugated streptavidin (Amersham, Amersham UK). Similarly prepared extracts of *E. coli* carrying pUK-IL-6 were used as positive control. Of the mAb CLB.IL-6/8 positive/mAb CLB.IL-6/ 16 negative mutants the nucleotide sequence of the XbaI-BanII fragment was subsequently determined. ELISA procedures and biotinylation of polyclonal antibodies have been described in detail by Brakenhoff et al., 1990, *supra* and Helle et al., *J. Immunol. Methods, 138*, 47 (1991).

As shown in Table 1, the mAb CLB.IL-6/16 epitope is disrupted by single substitution of Gln_{155}, Asn_{156}, Trp_{158}, and Thr_{163}. The double and triple substitution

mutants suggest that residues Ala_{154}, Leu_{159}, Gln_{160} and Met_{162} might also be important for the mAb CLB.IL-6/16 epitope.

Table 1. Bioactivity of rhIL-6 mutants that do not bind to mAb CLB.IL-6/16

TABLE 1

hIL-6 Variant	B9 Assay (U/μg)[a]	CESS Assay (U/μg)[a]
mature rhIL-6	6×10^6	1×10^4
$Gln_{155}His$	5×10^6	3×10^4
$Asn_{156}Lys$	1×10^6	6×10^4
$Trp_{158}^{*}(Gln)$	5×10^5	4×10^2
$Trp_{158}Gly$	4×10^6	4×10^3
$Trp_{158}Arg$	1×10^6	5×10^3
$Thr_{163}Pro$	2×10^6	< 20
$Ala_{154}Glu$, $Gln_{160}His$	5×10^5	not detectable
$Gln_{155}His$, $Gln_{157}Pro$	6×10^6	2×10^3
$Trp_{158}Cys$, $Met_{162}Ile$	6×10^4	$< 10^4$
$Trp_{158}Arg$, $Ser_{170}Asn$	5×10^6	2×10^3
$Gln_{160}Glu$, $Thr_{163}Pro$	3×10^6	< 20
$Ile_{137}Leu$, $Leu_{159}Arg$, $Met_{162}Ile$	1×10^7	5×10^3

[a]Concentration and biological activity in B9 and CESS assay of SDS-extracts of rhIL-6 mutants that bound to mAb CLB.IL-6/8 but not to mAb CLB.IL-6/16 were measured as described above. 1 unit is the amount of variant giving half-maximal stimulation in each assay. Values derived from one of two assays are shown.

C. Bioactivity of hIL-6 Mutants

The biological activity of crude extracts of various mutant proteins was subsequently measured both in the B9 assay and on IgG1 production by CESS cells. The B9 assay measures the murine hybridoma growth factor activity of rhIL-6 and variants as described in Aarden et al., *Eur. J. Immunol., 17,* 1411 (1987) and Helle et al., *Eur. J. Immunol., 18,* 1535 (1988). The CESS assay measures B-cell stimulatory factor-2 activity of rhIL-6 variants essentially as described by Poupart et al., *EMBO J., 6,* 1219 (1987). Briefly, CESS cells (6 x 10^3 cells/200 µl well in 96-well flat-bottom microtiterplates, in IMDM-5% FCS-Trf) were incubated for 4 days with serial dilutions of rhIL-6 or rhIL-6 variant containing samples in triplicate. IL-6 induced IgG1 production by the cells was subsequently measured in a sandwich ELISA by using a mouse mAb specific for human IgG1 (MH161-1M, Department of Immune Reagents, Central Laboratory of the Netherlands Red Cross Blood Transfusion Service (CLB), Amsterdam, The Netherlands) in combination with a horseradish peroxidase conjugated murine mAb specific for human IgG (MH16-1 ME, CLB) with a human serum as standard (H00-1234, CLB). ELISA procedures were as described above.

To measure the bioactivity of the mAb CLB.IL-6/8 positive/mAb CLB.IL6/16 negative mutants, overnight cultures of *E. coli* DH5α carrying the mutant constructs were diluted 1:50 in 250 ml LC amp medium and subsequently cultured to an OD550 of 1.5. Bacteria were harvested by centrifugation, resuspended in 5 ml lysis buffer (PBS, 1% Tween-20, 10 mM EDTA, 2 mM PMSF) and lysed by sonication. To solubilize rhIL-6 containing inclusion bodies, SDS was subsequently added to 1%. After 1 hour incubation at room temperature, SDS-insoluble material was removed by centrifugation (15 minutes at 13,000 g).

Bioactivity of this SDS solubilized material was directly measured in the B9 and CESS assays starting from a 1/1000 dilution. The hIL-6 variant concentration of these preparations was determined by means of a competitive inhibition radioimmunoassay (RIA) with IL-6 specific mAb CLB.IL-6/7 coupled to Sepharose 4B (Pharmacia LKB) and ^{125}I-rhIL-6 HGF7, in the presence of 0.1% SDS. Unlabelled rhIL-6 HGF7 served as a standard. mAb CLB.IL-6/7 binds heat and SDS denatured

IL-6 and recognizes hIL-6 residues Thr_{143}-Ala_{146} as determined by pepscan analysis (see Fontaine et al., *Gene, 104,* 227 (1991) and Arcone et al., *FEBS Letters, 288,* 197 (1991)).

As shown in Table 1, all mutants were biologically active in the murine B9 hybridoma proliferation assay. However, although very active in the B9 assay, no activity could be detected for the rhIL-6 $Thr_{163}Pro$ (rhIL-6 $T_{163}P$) single-mutant and rhIL-6 $Gln_{160}Glu$, $Thr_{163}Pro$ (rhIL-6 $Q_{160}E$, $T_{163}P$) double-mutant preparation on human CESS cells. (The nomenclature X_nY following a protein indicates that amino acid X at residue n has been replaced by amino acid Y, where X and Y are the commonly used three-letter or one-letter abbreviations for the naturally-occurring amino acids.).

D. Expression and Purification of Two hIL-6 Mutants

To confirm the role of the two mutants active in the B9 assay and inactive in the CESS assay described above, the hIL-6 cDNA inserts from the vectors pUK-IL-6 $T_{163}P$ and pUK-IL-6 $Q_{160}E$, $T_{163}P$ were removed with NcoI and BamHI and subcloned in NcoI-BamHI digested pET8c. Plasmid DNA was prepared from *E. coli* DH5α carrying the pET8c constructs and transformed to *E. coli* BL21 (DE3). *E. coli* BL21 (DE3) carrying these expression plasmids were subsequently cultured to an OD550 of 0.6 in LC amp medium and expression was induced by addition of 0.5 mM IPTG (Sigma). After a 3 hour induction period, the bacteria were harvested by centrifugation and the hIL-6 variants were purified essentially as described by Arcone et al., *Eur. J. Biochem., 198,* 541 (1991), with some modifications. Briefly, following centrifugation, bacteria were resuspended in 1/20 of the culture volume 10 mM Tris-HCl pH 7.4, 2 mM PMSF and frozen at -20°C. Following thawing bacteria were lysed by sonication. The sonicate was then applied upon a sucrose cushion (40% sucrose, 10 mM Tris-HCl pH 7.4) and centrifuged for 1 hour at 47,000 g. Pelleted inclusion bodies were subsequently washed once with PBS, 0.5% Tween-20, 10 mM EDTA, 2 mM PMSF and dissolved in 6 M guanidine-HCl, 25 mM Tris-HCl pH 7.4 (0.4 g wet weight/liter). Following two times dialysis against 20 volumes of 25 mM Tris-HCl pH 8.5, aggregates were removed by centrifugation for 1 hour at 11,000 g and the dialysate was concentrated 30-fold with an Amicon YM10 Filtration unit (Amicon Corp., Danvers, MA). The concentrate was then directly applied on a fast Q

Sepharose anion exchange column attached to a FPLC (Pharmacia LKB). Bound rhIL-6 variants were subsequently eluted with a linear gradient of NaCl in 25 mM Tris-HCl pH 8.5 and eluted at approximately 100 mM NaCl. The variants were subsequently sterile filtered and stored at -70°C. Protein concentration was determined both by measuring the optical density of the preparations and by the Bradford method (*Anal. Biochem., 72,* 248 (1976)) using BSA as a standard. Bradford and OD_{280} correlated best when assuming the OD_{280} of a 10 mg/ml solution of hIL-6 is 10.

Figure 1 shows a Coomassie blue stained SDS-polyacrylamide gel of the mutant preparations. The variants migrated with approximately the same molecular weight as mature rhIL-6. After the final purification step, two bands were observed in the rhIL-6 $Q_{160}E$, $T_{163}P$ preparation. Following Western blotting, both bands were recognized by an IL-6 specific mAb, suggesting that the lower band is a degradation product of the upper band (data not shown).

E. Bioactivity of Two hIL-6 Mutants

The bioactivity of these two mutants were tested both in the CESS assay and in two other available bioassays for IL-6: the HepG2 assay and the XG-1 assay.

The HepG2 assay measures the hepatocyte stimulating activity of rhIL-6 variants through the induction of C1 esterase inhibitor (C1 inh.) production by HepG2 cells as described by Zuraw et al., *J. Immunol., 265,* 12664 (1990). Following culturing to confluency (5×10^5 cells in 0.5 ml wells (Costar) in Iscove's Modified Dulbecco's Medium (IMDM) supplemented with 5% FCS, 5×10^{-5} M 2-ME, penicillin (100 IU), streptomycin (100 µg/ml) and human transferrin (20 µg/ml; Behringwerke, Marburg, Germany) (IMDM-5% FCS-Trf), HepG2 cells were washed twice and stimulated with serial dilutions of rhIL-6 or rhIL-6 mutants for 48 hours in the same medium in duplicate. In some experiments, cells were washed again after 24 hours and the stimulus was repeated for 24 hours. This procedure results in a higher

stimulation index. After the incubation period, C1 inh. synthesis was subsequently measured by sandwich RIA with anti-C1 inh. mAb RII coupled to Sepharose 4B and ^{125}I-labelled sheep polyclonal anti-C1 inh. IgG with normal human plasma as a standard as described in Nuijens et al., *J. Clin. Invest., 84,* 443 (1989) and Eldering et al., *J. Biol. Chem., 263,* 11776 (1988).

The XG-1 assay measures IL-6 activity on human myeloma cell line XG-1 essentially as described in Jourdan et al., *J. Immunol., 147,* 4402 (1991). Briefly, the cells were washed twice, incubated in IMDM-5% FCS-Trf for 4 hours at 37°C and then washed again. 10^4 cells/well in 200 µl IMDM-5% FCS-Trf in 96-well flat-bottom microtiterplates were subsequently incubated in triplicate with serial dilutions of rhIL-6 or rhIL-6 variants for 3 days. Following this culture period, proliferation of the cells was measured by labelling the cells with 7.4 kBq of [^{3}H]Thymidine (74 Gbq/mmol) for 4 hours and counting the radioactivity incorporated in the nuclei.

Figure 2a-c shows representative dose response curves of the mutants in three different assays with human cell lines. In Table 2, the specific activities of the mutants in the human assays is depicted, together with the specific activities in the (murine) B9 assay, relative to that of wild-type rhIL-6 HGF7. In the experiment in Figure 2a is shown that also the purified rhIL-6 $Q_{160}E$, $T_{163}P$ double-mutant did not induce IgG1 synthesis by the CESS cells. In some experiments however (see e.g. Figure 3a), a small increase in background IgG1 production was observed. As shown in Figure 2b, a weak induction of the acute phase protein C1 esterase inhibitor (C1 inh.) was reproducibly observed at high concentrations of the variant, with a strongly reduced plateau level as compared to wild-type rhIL-6 HGF7. However, although the specific activity of the double-mutant in inducing proliferation of the human myeloma cell line XG-1 (see Jourdan et al., *supra* and Figure 2c) was approximately 1,000-fold reduced as compared to wild-type hIL-6, almost the same plateau level was reached. On B9 cells the specific activity of rhIL-6 $Q_{160}E$, $T_{163}P$ was only 10-fold reduced (Table 2). RhIL-6 $T_{163}P$ was more active than the double-mutant in all assays, with a reduced plateau in CESS and HepG2 assays. The activity of the rhIL-6 $Q_{160}E$, $T_{163}P$ mutant on

XG-1 cells was not due to contamination by wild-type rhIL-6 because it could be inhibited by mAb CLB.IL-6/8, but not by mAb CLB.IL-6/16 (data not shown).

Table 2. Specific activities (U/μg) of purified rhIL-6 variants in IL-6 bioassays

TABLE 2

hIL-6 Variant	B9 ($x10^{-5}$)	CESS ($x10^{-3}$)	HepG2	XG-1 ($x10^{-4}$)
mature rhIL-6	11 ± 3	5.2 ± 0.3	542 ± 209	12 ± 0.3
HGF7	6 ± 2	4.7 ± 1.2	666 ± 165	4.9 ± 2.5
$Thr_{163}Pro$	2.1 ± 0.3	0.003 ± 0.003	11 ± 10	0.024 ± 0.0175
$Gln_{160}Glu$, $Thr_{163}Pro$	0.9 ± 0.4	< 0.001	< 0.1	0.004 ± 0.002

F. IL-6 Receptor Antagonistic Activity of Two hIL-6 Mutants

We tested these mutants for ability to antagonize the activity of wild-type rhIL-6 HGF7 on the cell lines. In Figure 3a and b is shown that rhIL-6 $Q_{160}E$, $T_{163}P$ completely inhibited the wild-type hIL-6 activity on CESS and HepG2 cells. In these experiments, 50% inhibition of IL-6 activity in CESS and HepG2 assays was observed with approximately 50 ng/ml and 1 μg/ml of rhIL-6 $Q_{160}E$, $T_{163}P$, respectively, corresponding to 20 and 200-fold the concentration of rhIL-6 HGF7 used to stimulate the cells. 100% inhibition was observed when the double-mutant was used in respectively 1,000 and 3,600-fold excess over wild-type. No inhibitory effects were observed on XG-1 cells. Of rhIL-6 $T_{163}P$ no antagonistic activity could be detected (data not shown). Figure 4 shows that the inhibitory effect of rhIL-6 $Q_{160}E$, $T_{163}P$ on IL-6 activity in the HepG2 assay could be reversed by high concentrations of rhIL-6 HGF7, suggesting competitive inhibition of IL-6 receptor binding by rhIL-6 $Q_{160}E$, $T_{163}P$. A similar result was found with CESS cells (data not shown).

HepG2 cells can synthesize C1 inh. in response to both IL-6 and IFN-γ via separate mechanisms (see Zuraw et al., *supra*). To further demonstrate the specificity of inhibition by the double-mutant, we tested whether rhIL-6 $Q_{160}E$, $T_{163}P$ could inhibit IFN-γ induced C1 inh. synthesis by the HepG2 cells. As shown in Figure 5, the C1 esterase inhibitor synthesis induced by 5 ng/ml of rhIL-6 HGF7 was inhibited to background levels, whereas the C1 inh. synthesis induced by 1 ng/ml of IFN-γ was unimpaired.

G. IL-6R Binding of Two hIL-6 Mutants

The fact that the rhIL-6 $Q_{160}E$, $T_{163}P$ could still be recognized by site I-specific mAb CLB.IL-6/8 and that it could antagonize wild-type IL-6 activity on CESS and HepG2 cells suggested that the 80 kDa binding site was still intact. To test this hypothesis binding of this variant to NIH-3T3 fibroblasts transfected with an expression vector encoding the 80 kDa IL-6 receptor (see Rose-John et al., *J. Biol. Chem., 266,* 3841 (1991)) was compared to that of wild-type rhIL-6 in a competitive inhibition assay. Figure 6 shows that the double-mutant was approximately 4-fold less efficient in inhibiting binding of ^{125}I-rhIL-6 to the cells, than rhIL-6 HGF7.

Example 2

Preparation of hIL-6 Variants

With Mutations in the Region Glu52-Glu60

A. Preparation of hIL-6 Variants

A panel of hIL-6 variants was constructed in which residues Glu_{52}, Ser_{53}, Ser_{54}, Lys_{55}, Glu_{56}, Leu_{58} and Glu_{60} were individually replaced by alanine. Site directed mutagenesis on the hIL-6 cDNA was done by a two step PCR mutagenesis technique (see Landt et al., *Gene, 96*:125 (1990)). To facilitate subcloning of PCR fragments, a unique EcoRI site was introduced into the hIL-6 coding region by making two silent substitutions in the codons of Glu_{94} and Phe_{95} (GAGTTT --> GAATTC). The unique EcoNI and EcoRI sites were used for subcloning of PCR fragments with alanine

substitutions of residues Glu_{52}, Ser_{53}, Ser_{54}, Lys_{55}, Glu_{56}, Leu_{58} and Glu_{60}, in the T7 promotor-vector pRSET6D (T. Stoyan, Institut für Biochemie, RWTH, Aachen, FRG). If possible, restriction sites were concomitantly introduced with the alanine substitutions, to facilitate identification of positive clones. Primers were synthesized on an Applied Biosystems DNA synthesizer type 381A (Warrington, GB). The mutagenesis primers (antisense) used were: (Glu_{52}Ala) 5'-CTT TGC TGC TTG CAC ACA TGT T-3', SEQ ID NO: 6; (Ser_{53}Ala) 5'-GTG CCT CTT TGG AAG CTT CAC ACA TGT TA-3', SEQ ID NO: 7; (Ser_{54}Ala) 5'-GTG CCT CTT TAG CGC TTT CAC AC-3', SEQ ID NO: 8; (Lys_{55}Ala) 5'-CCA GTG CCT CAG CGC TGC TTT CAC-3', SEQ ID NO: 9; (Glu_{56}Ala) 5'-CTG CCA GTG CGG CCT TGC TGC TTT C-3', SEQ ID NO: 10; (Leu_{58}Ala) 5'-GTT GTT TTC TGC AGC TGC CTC TTT GC-3', SEQ ID NO: 11; and (Glu_{60}Ala) 5'-GTT CAG GTT GTT GGC CGC CAG TGC CTC-3', SEQ ID NO: 12. The integrity of the constructs was verified by restriction enzyme digestion and nucleotide sequence analysis on dsDNA of the EcoNI-EcoRI fragments with the dideoxy chain termination method, using the Sequenase kit (Biochemical Corporation, Cleveland, USA). For expression of the hIL-6 variants, the vectors were transformed into *E. coli* BL21 (DE3) and the proteins were purified from inclusion bodies and quantitated as described in Brakenhoff et al., *J. Biol., Chem., 269*:86 (1994).

B. Activity of the Variants in Bioassays

The biological activity of the mutant proteins was measured in three different bioassays for IL-6: induction of mouse B9 hybridoma proliferation and of XG-1 human myeloma cell proliferation, and inhibition of the proliferation of the human melanoma cell line A375. The B9 and XG-1 assays were conducted essentially as described above in Example 1. IL-6 can also inhibit the proliferation of the human melanoma cell line A375 (Te Velde et al., *Int. J. Cancer, 50*:746-751 (1992)). Briefly, the A375 assay was conducted as follows. A375 cells were maintained in Iscove's Modified Dulbecco's Medium supplemented with 5% v/v fetal calf serum, 5 x 10^{-5} M 2-mercaptoethanol, 100 IU penicillin, 100 μg/ml streptomycin, 20 μg/ml

human transferrin (Behringwerke, Marburg, FRG). To test the ability of hIL-6 mutant proteins to inhibit proliferation of A375 cells, the cells were incubated in 96-well flat-bottomed microtiter plates (4 x 10^3 cells/well in triplicate for 72 h in the presence of serial dilutions of the samples to be tested. After this culture period, the cells were pulse-labeled with 7.4 kBq of [^{3}H]thymidine (74 Gbq/mmol) for 5 h, detached with 10 mM EDTA and the radioactivity incorporated in the nuclei was measured as described in Brakenhoff et al., *J. Biol., Chem., 269*:86 (1994).

The bioactivity data is shown in Table 3 below. In the B9 assay, all of the variants (mutants) had activity similar to that of wild type hIL-6 (Table 3). In the human cell assays (XG-1 and A375), only the $Leu_{58}Ala$ mutant protein displayed a consistent ~ 5-fold reduction in specific activity. The full activity of $Leu_{58}Ala$ on B9 cells suggests that the reduced activity on human cells was probably not due to a perturbation of the overall tertiary structure of the $Leu_{58}Ala$ protein. The $Leu_{58}Ala$ variant also bound to conformation specific mAbs CLB.IL6/8, CLB.IL6/15 and CLB.IL6/16 with affinity comparable to that of wthIL-6 and the other variants.

Table 3

Characteristics of IL-6 mutant proteins with alanine substitutions of residues Glu52-Glu60[a].

	Bioassay[b]			Receptor binding[c]		mAb binding[c]		
	B9	XG-1	A375	IL-6Rα	gp130	IL6/8	IL6/15	IL6/16
wthIL-6	100	100	100	100	100	100	100	100
$E_{52}A$	97	167	42	44	105	44	58	56
$S_{53}A$	54	194	30	78	110	40	54	59
$S_{54}A$	122	173	70	59	115	33	41	57
$K_{55}A$	125	145	43	40	115	58	49	57
$E_{56}A$	152	196	82	75	120	76	88	82
$L_{58}A$	93	20	18	94[d]	16[e]	31	60	75
$E_{60}A$	108	75	30	93	110	47	51	61

a) The responses of the hIL-6 mutants in the various assays are expressed as the percentage of the response of wild type hIL-6 (set at 100%) and are derived from EC_{50} values (the effective concentration that gives a half-maximal response). Intra-assay SD values were <20% of the average for all assays. Interassay variation for a particular mutant was generally less than a factor of 3. A 2-3 fold difference in EC_{50} value between the mutants is therefore not considered significant.

b) The biological activity values of the hIL-6 mutants in the B9, XG-1 and A375 assays represent the average of two experiments. The average EC_{50} was 1 pg/ml in the B9 assay, 20 pg/ml in the XG-1 assay, and 0.2 ng/ml in the A375 assay, respectively.

c) The receptor-binding and mAb-binding values of the hIL-6 mutants are the average of two experiments, except for the gp130 binding assay, which represents the averages from duplicate measurements of one experiment. The average EC_{50} of wthIL-6 was 100 ng/ml in the IL-6RA binding assay, 10 ng/ml in the gp130 binding assay, and was 2, 2, and 0.5 ng/ml in the mAb CLB.IL6/8, mAb CLB.IL6/15 and mAb CLB.IL6/16 ELISAs, respectively.

d) The receptor-binding value represents an average of four assays.

e) The EC_{50} could not be determined precisely, as no maximal response was obtained.

C. Receptor Binding Activity of the Variants

The capacity of hIL-6 and the hIL-6 mutant proteins to induce IL-6Rα-dependent association with gp130 was measured in an ELISA with immobilized sIL-6Rα and a gp130-IgG1 fusion protein consisting of the extracellular domain of gp130 fused to the hinge region and last two constant region domains of human IgG1 as described in De Hon et al., *J. Exp. Med., 180*:2395 (1994). The results are shown above in Table 3. No significant differences from wild type hIL-6 were observed in the capacity of the mutants to bind to soluble (s)IL-6Rα. However, the $Leu_{58}Ala$ mutant showed a reduced response in the sIL6Rα-dependent binding to sgp130.

These results reveal that Leu_{58} is involved in IL-6R-dependent binding to gp130. However, it seems likely that other residues besides Leu_{58} in this region are involved in gp130 interaction, because single substitution of Leu_{58} only resulted in an approximately 5-fold reduction in activity and in IL-6R-dependent gp130 binding.

Example 3

Preparation of Improved hIL-6 Receptor Antagonists

A. Preparation of the Antagonist hIL-6.L58A/Q160E/T163P/F171L/S177R

An hIL-6 mutein having five mutations, $Leu_{58}Ala$, $Gln_{160}Glu$, $Thr_{163}Pro$, $Phe_{171}Leu$, and $Ser_{177}Arg$, was prepared by recombinant means. The mutein is the expression product of a vector named pRSET6D-IL6.L58A/Q160E/T163P/F171L/S177R, which was prepared as follows. The T7 promotor vector pRSET6D (T. Stoyan, Institut für Biochemie, RWTH, Aachen, FRG) was used for expression of hIL-6 mutant proteins. pRSET6D-IL6.L58A/Q160E/T163P/F171L/S177R was constructed in three steps. The construction of the expression vector pET8c-IL-6.Q160E/T163P has been described in Brakenhoff et al., *J. Biol., Chem., 269*:86 (1994). The complete IL-6.Q160E/T163P NcoI-BamHI cDNA fragment was subcloned in NcoI-BamHI digested pRSET6D. To

construct pRSET-IL-6.Q160E/T163P/F171L/S177R, an XbaI-Bsu36I fragment encoding the $F_{171}L$ and $S_{177}R$ substitutions was created with PCR technology by using pRSET6D-IL-6.Q160E/T163P as a template and subcloned into XbaI- Bsu36I digested pRSET6D-IL-6.Q160E/T163P as described in De Hon et al., *J. Exp. Med.* 180:2395 (1994). Briefly, PCR was carried out with Pfu polymerase (Stratagene, La Jolla, USA) following standard procedures (denaturation 1.5 min 95°C, annealing 1 min. 55°C, extension 1 min 72°C; 30 cycles) and the following primers: a sense primer carrying the XbaI site (5'-GAA TCT AGA TGC AAT AAC CAC C-3', SEQ ID NO: 13), and an antisense primer encoding the two substitutions and the Bsu36I site (5'-GAA GAG CCC TCA GGC TGC GCT GCA GGA ACT CCT TAA GGC TGC GCA GAA TG-3', SEQ ID NO: 14).

The vector pRSET6D-IL6.L58A encoding a hIL-6 variant with an alanine substitution at Leu_{58} was constructed using PCR mutagenesis techniques as described above in Example 2. Finally, pRSET6D-IL-6.L58A/Q160E/T163P/F171L/S177R was constructed by ligating an NcoI-XbaI hIL-6 cDNA fragment from pRSET6D-IL-6.L58A carrying the $L_{58}A$ mutation into NcoI-XbaI digested pRSET6D-IL-6.Q160E/T163P/F171L/S177R. The integrity of the constructs was verified by restriction enzyme digestion and nucleotide sequence analysis of dsDNA with the dideoxy chain termination method, using the Sequenase kit (Biochemical Corporation, Cleveland, USA).

For expression of the hIL-6 mutant protein, the pRSET6D-L58A/Q160E/T163P/F171L/S177R vector was transformed into *E.coli* JM109 (DE3) (Promega), the protein was purified to ~80% homogeneity from inclusion bodies, and was quantified by radioimmunoassay as described in Brakenhoff et al., *J. Biol. Chem.*, *269*:86 (1994).

C. Activity of the Antagonist in Bioassays

The bioactivity of the mutein was tested in the XG-1 and B9 assays. The wthIL-6 preparation used as a standard was mature recombinant human (rh) IL-6

(Ala_1-Met_{185}) purified from *E. coli* BL21 (DE3) carrying the pET8c-hIL-6 cDNA expression vector (Brakenhoff et al., *J. Biol. Chem, 269*:86 (1994).

The human XG-1 myeloma proliferation assay was performed essentially as described in Example 1 above. Briefly, before start of the assay the cells were washed twice, incubated for 3-4 h at 37°C without IL-6 in Iscove's Modified Dulbecco's Medium supplemented with 5% FCS, $5x10^{-5}$M 2-ME, penicillin (100-IU), streptomycin (100 μg/ml), human transferrin (20 μg/ml; Behringwerke, Marburg, FRG) (IMDM-5% FCS-Trf) and then washed again. 10^4 cells/well in 200 μl IMDM-5% FCS-Trf in 96-well flat-bottomed microtiterplates were subsequently incubated in triplicate with serial dilutions of wthIL-6 or hIL-6 variant for three days. Following this culture period, proliferation of the cells was measured by labeling the cells with 7.4 kBQ of [^{3}H]Thymidine (74 Gbq/mmol) for 4 hours and counting the radioactivity incorporated in the nuclei.

The mouse hybridoma proliferation (B9) assay was performed as described in Example 1 above and in Aarden et al., *Eur. J. Immunol., 17*:1411 (1987).

The results of the XG-1 assay are displayed in Figures 7-8. Figure 7 (wherein the closed circles represent the mutein's activity and the open diamonds represent wild type hIL-6 activity) shows that hIL-6-$L_{58}A/Q_{160}E/T_{163}P/F_{171}L/S_{177}R$ was inactive on XG-1 cells. Figure 8 (wherein the closed circles represent the activity of wild type hIL-6 alone and the open circles represent the activity of wild type hIL-6 with the mutein) shows that the addition of 10μg/ml of hIL-6-$L_{58}A/Q_{160}E/T_{163}P/F_{171}L/S_{177}R$ had an antagonistic effect on wild type hIL-6 activity on these cells. The antagonistic effect was reversible with increasing concentrations of wild type hIL-6, indicating that the mutein is a competitive antagonist and that the antagonistic effect is not due to aspecific toxicity.

The results of the B9 assay are displayed in Figure 9 (wherein the closed circles represent hIL-6-$L_{58}A/Q_{160}E/T_{163}P/F_{171}L/S_{177}R$ activity and the open diamonds represent wild type hIL-6 activity). hIL-6-$L_{58}A/Q_{160}E/T_{163}P/F_{171}L/S_{177}R$ had an approximately 1000-fold reduced specific activity on mouse B9 cells as compared to wild type hIL-6.

The results described above show that hIL-6-$L_{58}A/Q_{160}E/T_{163}P/F_{171}L/S_{177}R$ is almost completely inactive on XG-1 cells and furthermore exhibits antagonistic activity in the XG-1 assay. The 1000-fold reduction in hIL-6-$L_{58}A/Q_{160}E/T_{163}P/F_{171}L/S_{177}R$ activity on B9 cells has also not been previously reported for any other hIL-6 receptor antagonist (including those of the previous Examples 1 or 2). This synergistic decrease in activity is much higher than would be expected from combining the $L_{58}A$ mutation (which had almost full activity (93% of wild type) on B9 cells) with the $Q_{160}E$ and $T_{163}P$ mutations (the combination $Q_{160}E/T_{163}P$ mutant had only a 10-fold reduction in activity on B9 cells).

D. Receptor Binding Activity of the Antagonist

Binding to the IL-6 receptor IL-6Rα was evaluated using an ELISA in which the capacity of the hIL-6 mutant proteins to compete for binding of biotinylated wild type hIL-6 to immobilized soluble (s) IL-6Rα was measured. Briefly, purified wthIL-6 was biotinylated by using LC-biotin-N-hydroxysuccinimide ester (Pierce Chemical Co., Rockford, USA) following the manufacturer's instructions. Microtiter plates (Nunc, maxisorb, Roskilde, Denmark) were coated overnight with anti-IL-6Rα mAb MT18 (3 μg/ml in PBS, Hirata et al., *J. Immunol., 143*:2900 (1989)). The plates were washed with PBS, 0.02% Tween-20 (PT) and incubated for 2 h with conditioned medium of NIH-3T3 fibroblasts expressing the sIL-6Rα (Mackiewicz et al., *J. Immunol.*, 149:2021 (1992)). After washing, serial dilutions of the mutants, together with 8 ng/ml of biotinylated wthIL-6, were incubated in duplicate at room temperature till binding reached equilibrium (>2 h). The wells were subsequently emptied and bound biotinylated wthIL-6 was detected with poly-horseradish peroxidase streptavidin without further washing. Following washing with PT, bound poly HRP was finally detected with 3,3',5,5'-tetramethylbenzidine (TMB; Merck, Darmstadt, FRG) staining as described in Brakenhoff et al., *J. Biol. Chem, 269*:86 (1994).

The results are displayed in Figure 10 (wherein the closed circles represent and the open diamonds represent wild type hIL-6). hIL-6-$L_{58}A/Q_{160}E/T_{163}P/F_{171}L/S_{177}R$ binds with similar affinity (about 3-fold lower) as wild type hIL-6 to the soluble IL-

6Rα chain, as measured by its ability to compete for the binding of biotinylated IL-6 to this chain.

The reactivity of the mutein with IL-6 conformation-specific mAbs CLB.IL6/8, CLB.IL6/12 and CLB,IL.6/14 was also measured in sandwich ELISAs with the mAbs as coating antibody, serial dilutions of the mutant protein preparation and affinity purified biotinylated sheep polyclonal anti-rhIL-6 as detecting antibodies as described in Brakenhoff et al., *J. Biol. Chem, 269*:86 (1994). The mutein and wild type hIL-6 exhibited almost the same binding affinity for all three antibodies, showing that the overall tertiary structure of this protein is comparable to that of wild type hIL-6.

Numerous modifications and variations in the practice of the invention are expected to occur to those skilled in the art upon consideration of the foregoing description of the presently preferred embodiments thereof. Consequently, the only limitations which should be placed upon the scope of the present invention are those which appear in the appended claims.

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(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.25

(vi) CURRENT APPLICATION DATA:
(A) APPLICATION NUMBER: Unassinged
(B) FILING DATE: Even date herewith
(C) CLASSIFICATION:

(vii) PRIOR APPLICATION DATA:
(A) APPLICATION NUMBER:
(B) FILING DATE:

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(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 22 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

GAATCTAGAT GCAATAACCA CC 22

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 34 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

TGTCATGTCC TGCAGCCACT GGTTCTGTGC CTGC 34

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 34 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

CAGGCACAGA ACCAGTGGCT GCAGGACATG ACAA 34

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 21 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

CGAAGAGCCC TCAGGCTGGA C 21

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 185 amino acids
(B) TYPE: amino acid

(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

```
Ala Pro Val Pro Pro Gly Glu Asp Ser Lys Asp Val Ala Ala Pro His
1               5                  10                  15

Arg Gln Pro Leu Thr Ser Ser Glu Arg Ile Asp Lys Gln Ile Arg Tyr
            20                  25                  30

Ile Leu Asp Gly Ile Ser Ala Leu Arg Lys Glu Thr Cys Asn Lys Ser
        35                  40                  45

Asn Met Cys Glu Ser Ser Lys Glu Ala Leu Ala Glu Asn Asn Leu Asn
    50                  55                  60

Leu Pro Lys Met Ala Glu Lys Asp Gly Cys Phe Gln Ser Gly Phe Asn
65                  70                  75                  80

Glu Glu Thr Cys Leu Val Lys Ile Ile Thr Gly Leu Leu Glu Phe Glu
                85                  90                  95

Val Tyr Leu Glu Tyr Leu Gln Asn Arg Phe Glu Ser Ser Glu Glu Gln
            100                 105                 110

Ala Arg Ala Val Gln Met Ser Thr Lys Val Leu Ile Gln Phe Leu Gln
        115                 120                 125

Lys Lys Ala Lys Asn Leu Asp Ala Ile Thr Thr Pro Asp Pro Thr Thr
    130                 135                 140

Asn Ala Ser Leu Thr Thr Lys Leu Gln Ala Gln Asn Gln Trp Leu Gln
145                 150                 155                 160

Asp Met Thr Thr His Leu Ile Leu Arg Ser Phe Lys Glu Phe Leu Gln
                165                 170                 175

Ser Ser Leu Arg Ala Leu Arg Gln Met
            180                 185
```

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 22 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

CTTTGCTGCT TGCACACATG TT 22

(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 29 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

GTGCCTCTTT GGAAGCTTCA CACATGTTA 29

(2) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 23 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

GTGCCTCTTT AGCGCTTTCA CAC 23

(2) INFORMATION FOR SEQ ID NO:9:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 24 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

CCAGTGCCTC AGCGCTGCTT TCAC 24

(2) INFORMATION FOR SEQ ID NO:10:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 25 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

CTGCCAGTGC GGCCTTGCTG CTTTC 25

(2) INFORMATION FOR SEQ ID NO:11:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 26 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

GTTGTTTTCT GCAGCTGCCT CTTTGC 26

(2) INFORMATION FOR SEQ ID NO:12:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 27 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

GTTCAGGTTG TTGGCCGCCA GTGCCTC 27

(2) INFORMATION FOR SEQ ID NO:13:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 22 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

GAATCTAGAT GCAATAACCA CC 22

(2) INFORMATION FOR SEQ ID NO:14:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 50 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:14:

GAAGAGCCCT CAGGCTGCGC TGCAGGAACT CCTTAAGGCT GCGCAGAATG 50

WE CLAIM:

1. An interleukin-6 (IL-6) receptor antagonist comprising a modified human IL-6 protein or a receptor-binding fragment thereof comprising the following mutations: the amino acid at the position corresponding to residue 58 of mature hIL-6 protein is alanine, the amino acid at the position corresponding to residue 160 of mature hIL-6 protein is glutamic acid, the amino acid at the position corresponding to residue 163 of mature hIL-6 protein is proline, the amino acid at the position corresponding to residue 171 of mature hIL-6 protein is leucine, and the amino acid at the position corresponding to residue 177 of mature hIL-6 protein is arginine.

2. A pharmaceutical composition comprising a therapeutically effective amount of the IL-6 receptor antagonist of claim 1 and a pharmaceutically acceptable carrier.

3. The pharmaceutical composition of claim 2 wherein the carrier is an aqueous solution of saline.

4. The pharmaceutical composition of claim 2 wherein the carrier is an aqueous dextrose solution.

5. The pharmaceutical composition of claim 3 in unit dose form.

6. The pharmaceutical composition of claim 5 in a form suitable for injection.

7. A method for treating a disease manifested by the expression of IL-6, said method comprising administering to a patient in need of such treatment the pharmaceutical composition of claim 2.

8. The method of claim 7 wherein the patient is a human.

9. The method of claim 8 wherein the disease is sepsis.

10. The method of claim 8 wherein the disease is multiple myeloma.

11. An IL-6 receptor antagonist consisting of a modified human IL-6 protein or a receptor-binding fragment thereof having the following mutations: the amino acid at the position corresponding to residue 58 of mature hIL-6 protein is alanine, the amino acid at the position corresponding to residue 160 of mature hIL-6 protein is glutamic acid, the amino acid at the position corresponding to residue 163 of mature hIL-6 protein is proline, the amino acid at the position corresponding to residue 171 of mature hIL-6 protein is leucine, and the amino acid at the position corresponding to residue 177 of mature hIL-6 protein is arginine.

Figure 1

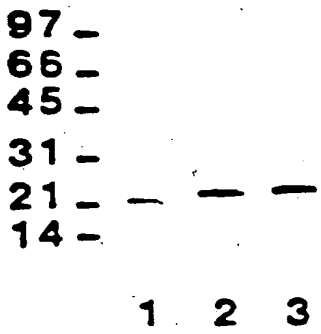


Figure 2A

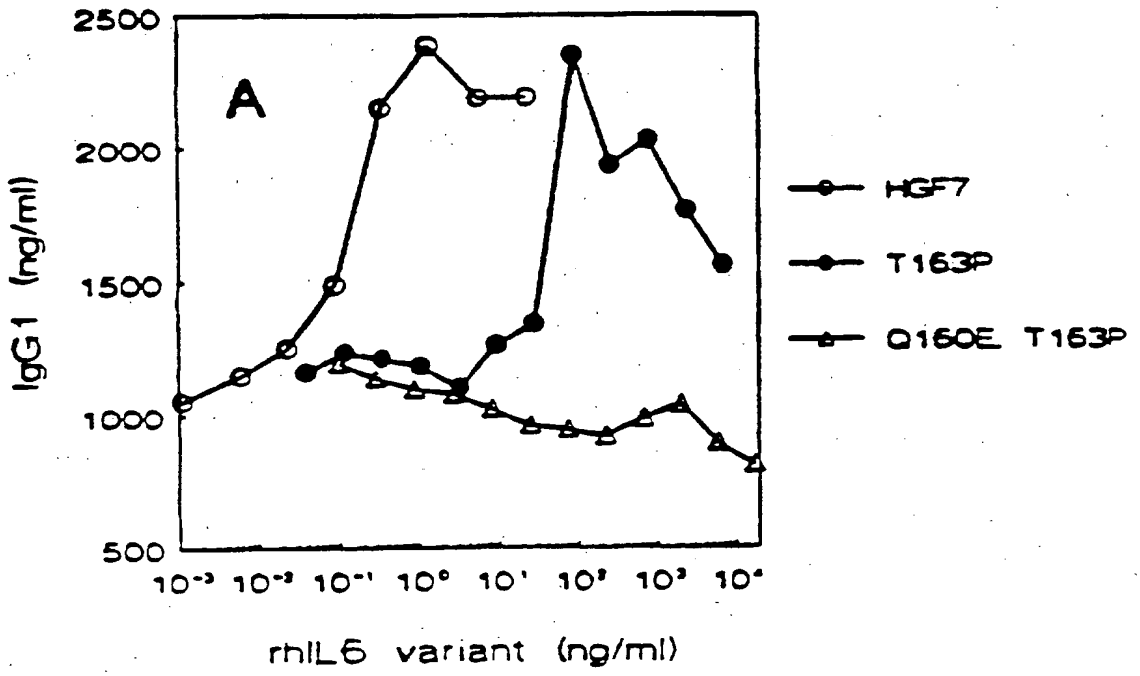

A
2500
2000
1500
1000
500
IgG1 (ng/ml)
10^{-3} 10^{-2} 10^{-1} 10^{0} 10^{1} 10^{2} 10^{3} 10^{4}
rhIL6 variant (ng/ml)
HGF7
T163P
Q160E T163P

Figure 2B

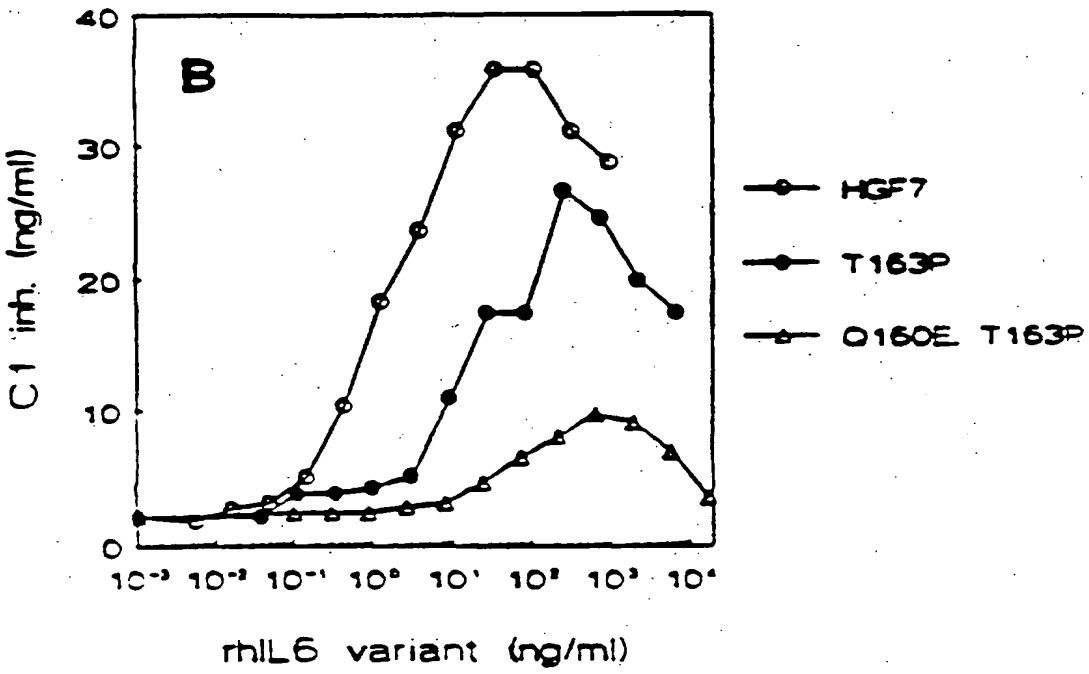

B
C1 inh. (ng/ml)
40
30
20
10
0
10⁻³ 10⁻² 10⁻¹ 10⁰ 10¹ 10² 10³ 10⁴
rhIL6 variant (ng/ml)
HGF7
T163P
Q160E T163P

Figure 2C

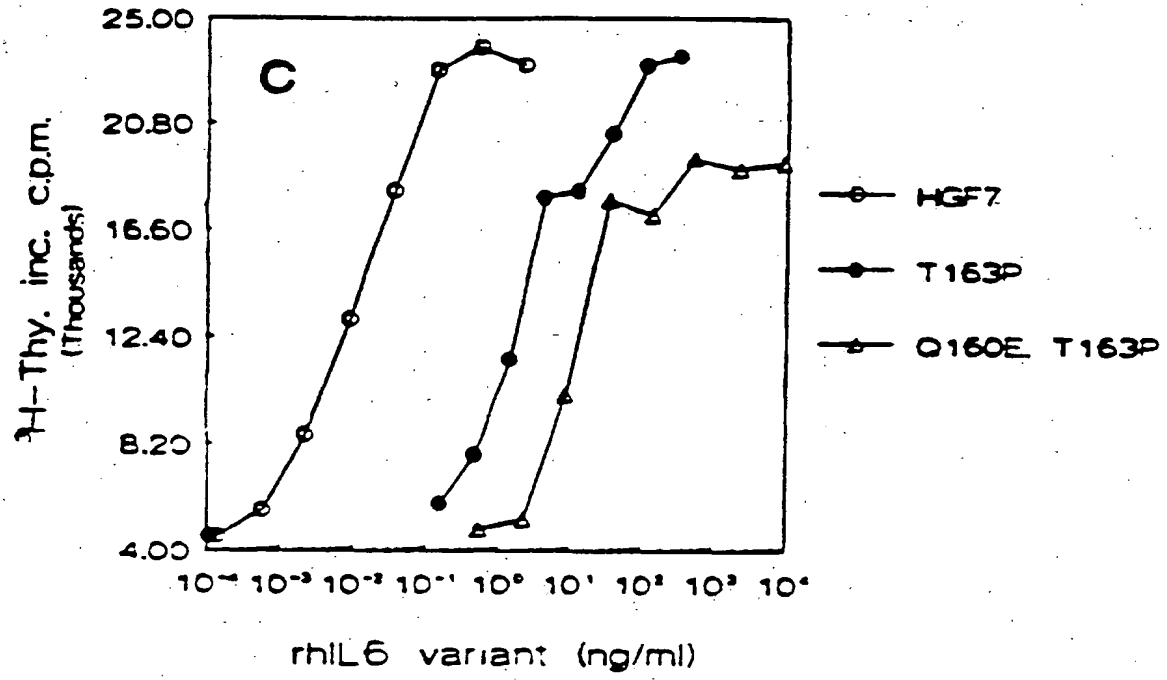

C
25.00
20.80
16.60
12.40
8.20
4.00
3H-Thy. inc. c.p.m. (Thousands)
10⁻⁴ 10⁻³ 10⁻² 10⁻¹ 10⁰ 10¹ 10² 10³ 10⁴
rhIL6 variant (ng/ml)
HGF7
T163P
Q160E T163P

Figure 3A

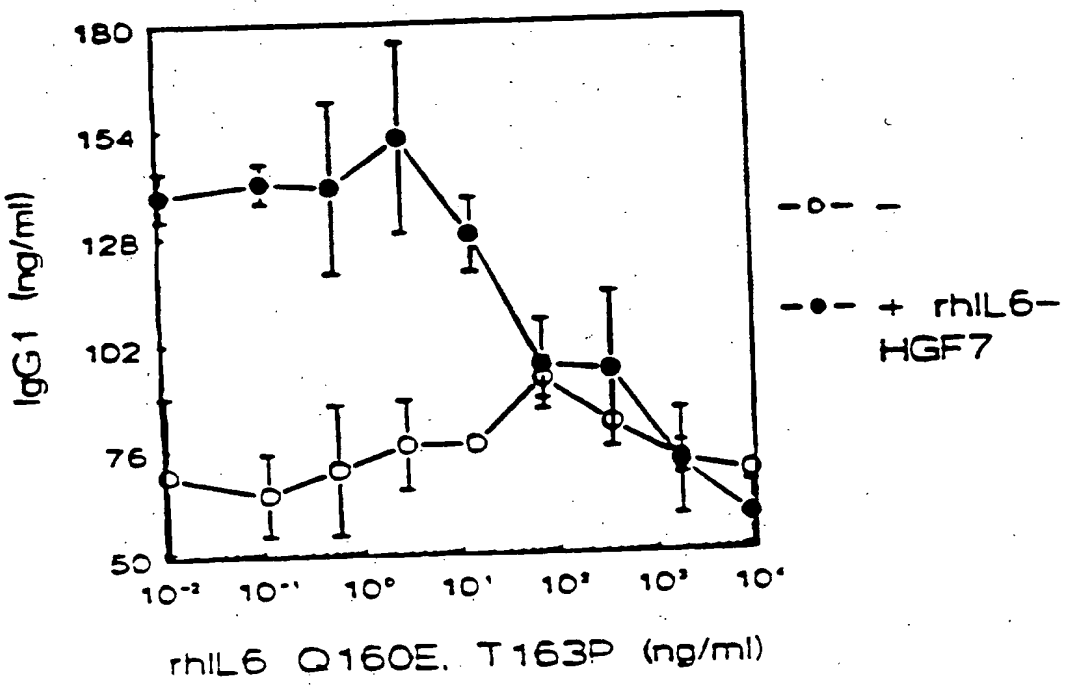

180
154
128
102
76
50
IgG1 (ng/ml)
10⁻²
10⁻¹
10⁰
10¹
10²
10³
10⁴
rhIL6 Q160E, T163P (ng/ml)
-o- -
-●- + rhIL6-HGF7

Figure 3B

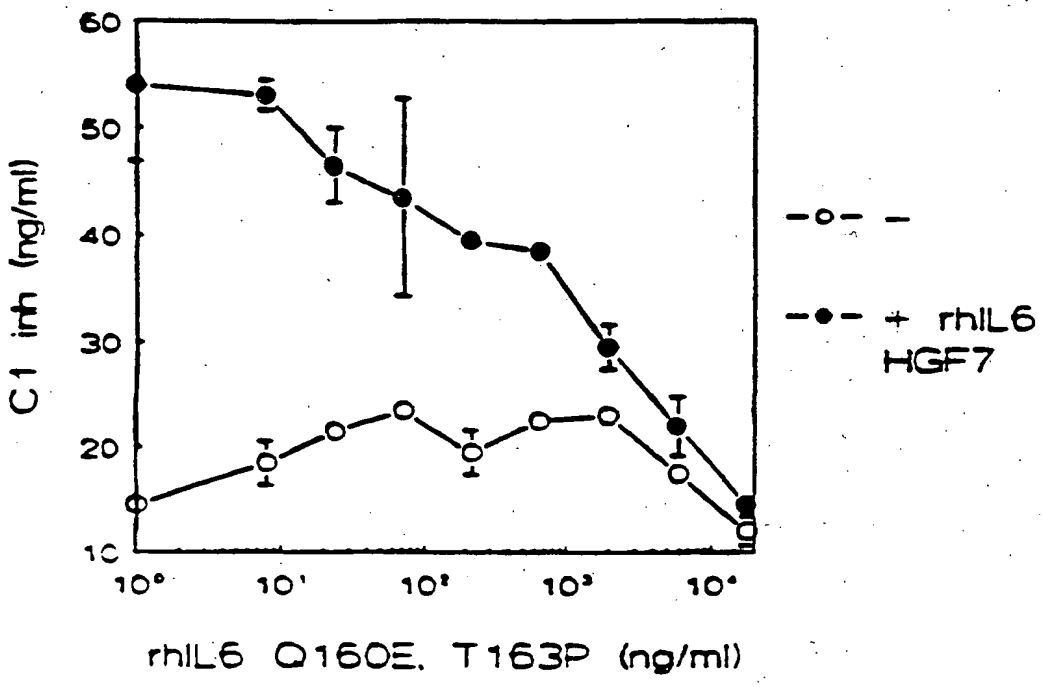

60
50
40
30
20
10
C1 inh (ng/ml)
10⁰
10¹
10²
10³
10⁴
rhIL6 Q160E, T163P (ng/ml)
– –
+ rhIL6
HGF7

Figure 4

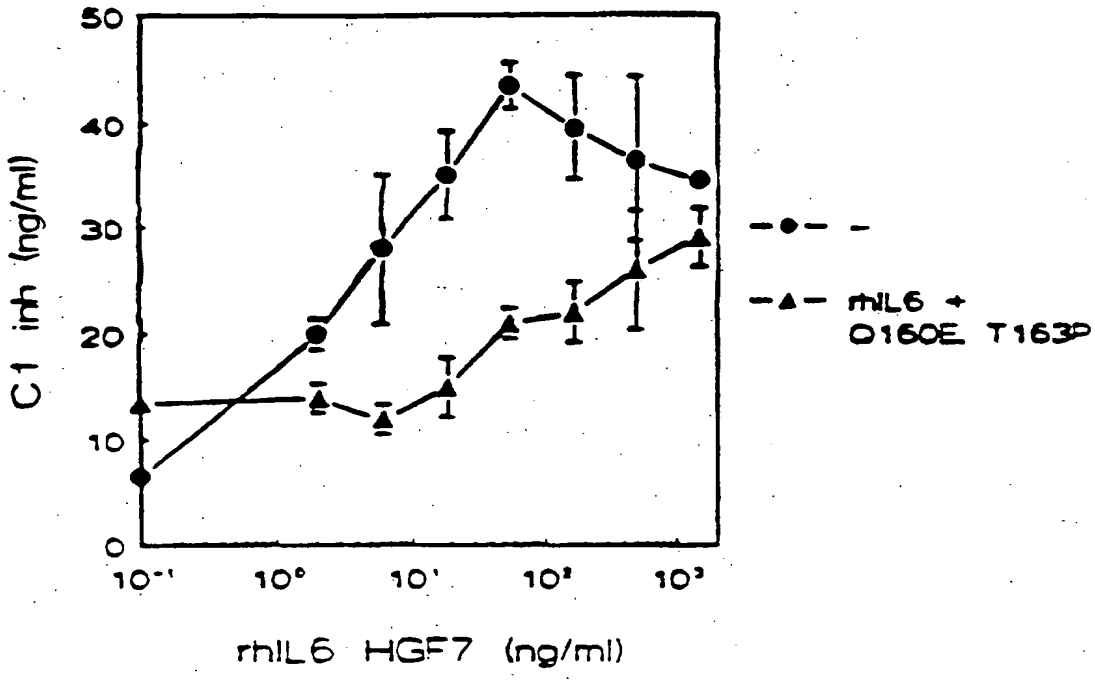

50
40
30
20
10
0
C1 inh (ng/ml)
10⁻¹
10⁰
10¹
10²
10³
rhIL6 HGF7 (ng/ml)
rhIL6 +
Q160E T163P

Figure 5

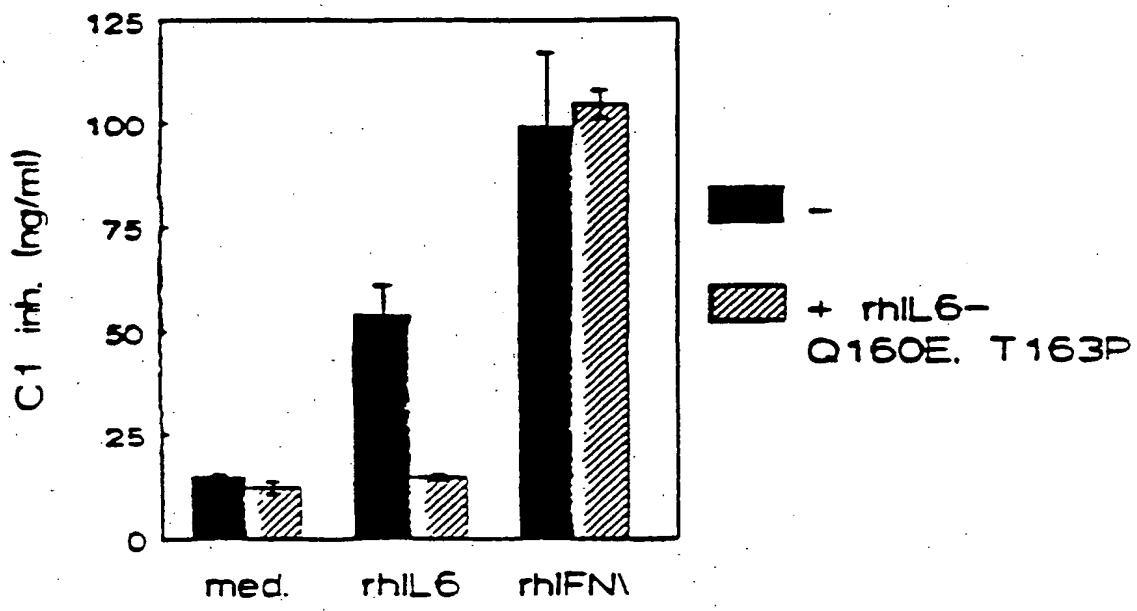

125
100
75
50
25
0
C1 inh. (ng/ml)
med.
rhIL6
rhIFN\
-
+ rhIL6-
Q160E. T163P

Figure 6

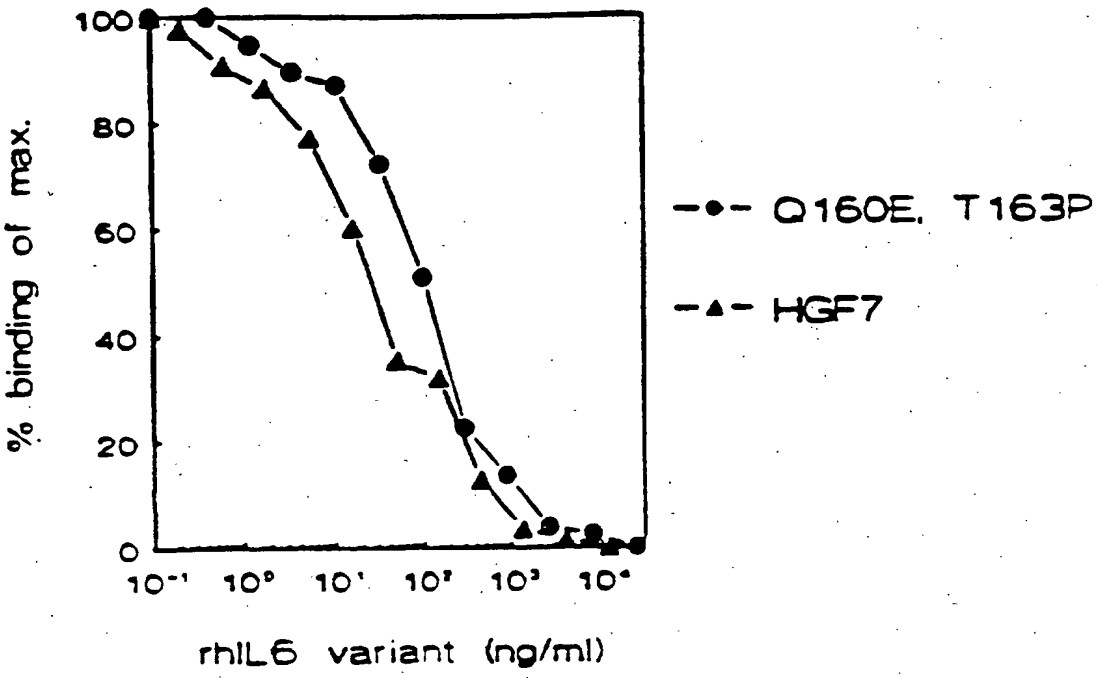

100
80
60
40
20
0
% binding of max.
10⁻¹ 10⁰ 10¹ 10² 10³ 10⁴
rhIL6 variant (ng/ml)
Q160E, T163P
HGF7

Figure 7

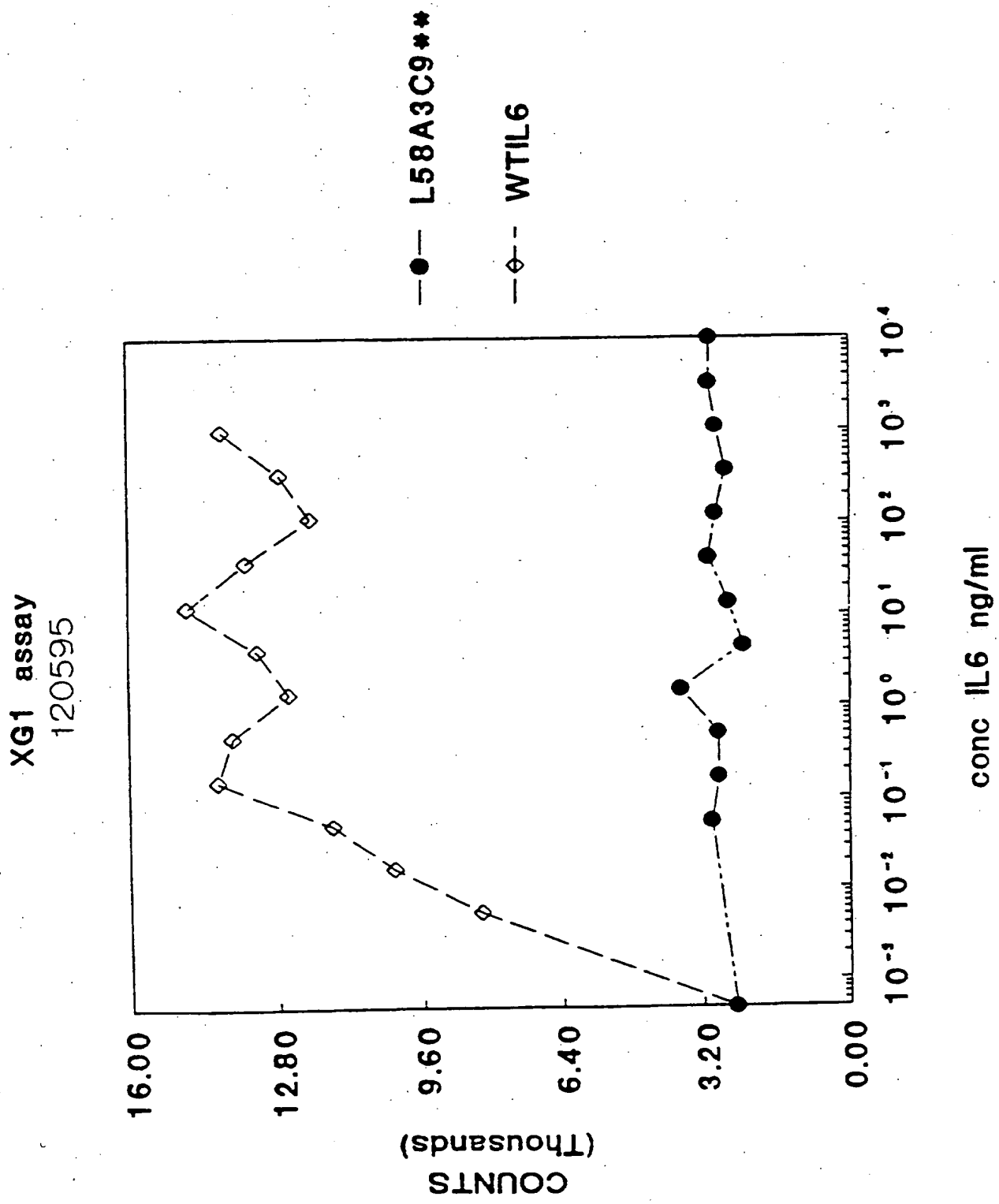

L58A3C9**
WTIL6
XG1 assay
120595
COUNTS
(Thousands)
16.00
12.80
9.60
6.40
3.20
0.00
10^{-3} 10^{-2} 10^{-1} 10^{0} 10^{1} 10^{2} 10^{3} 10^{4}
conc IL6 ng/ml

Figure 8

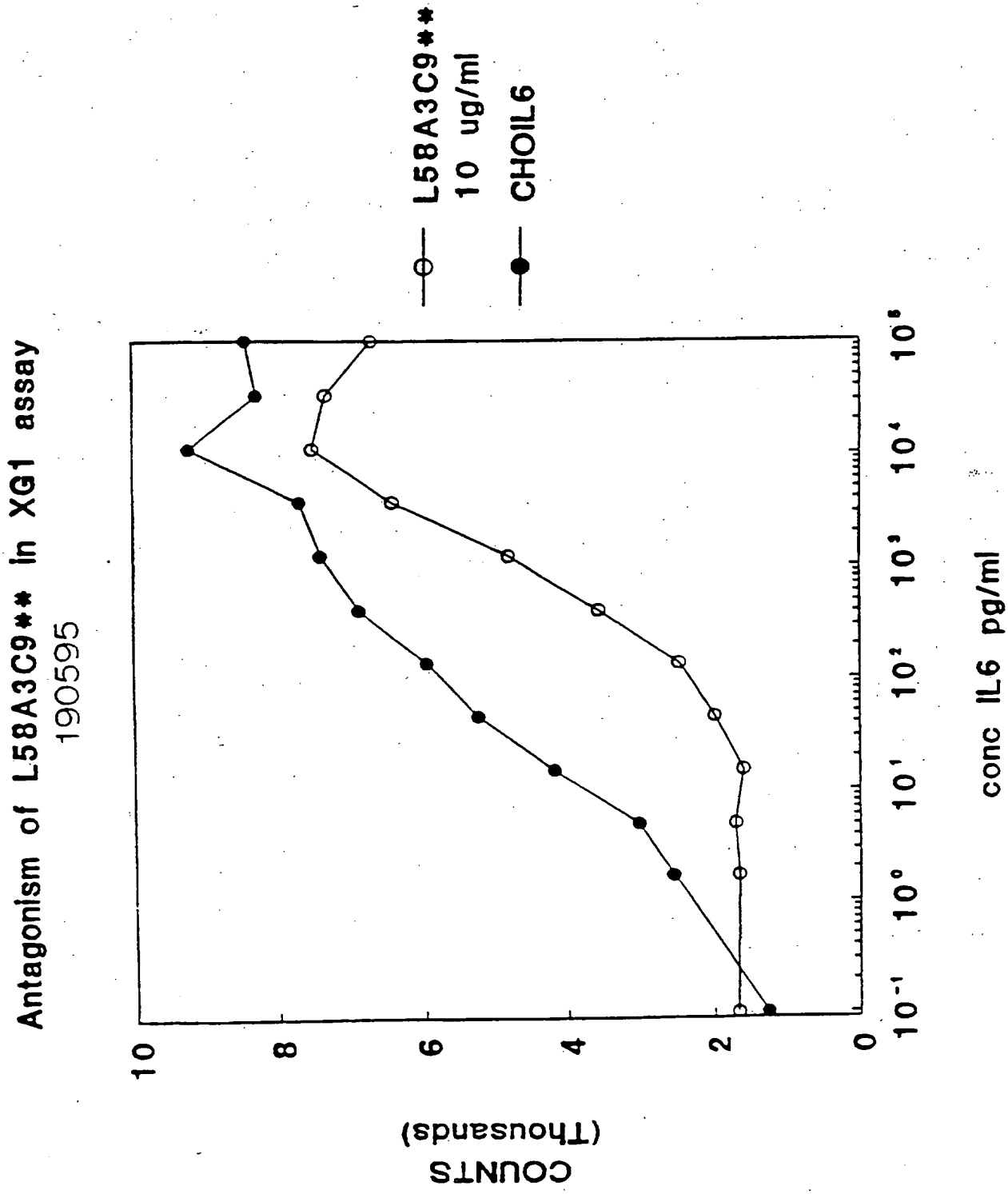

Antagonism of L58A3C9** in XG1 assay
190595
L58A3C9**
10 ug/ml
CHOIL6
COUNTS
(Thousands)
0
2
4
6
8
10
10⁻¹
10⁰
10¹
10²
10³
10⁴
10⁵
conc IL6 pg/ml

Figure 9

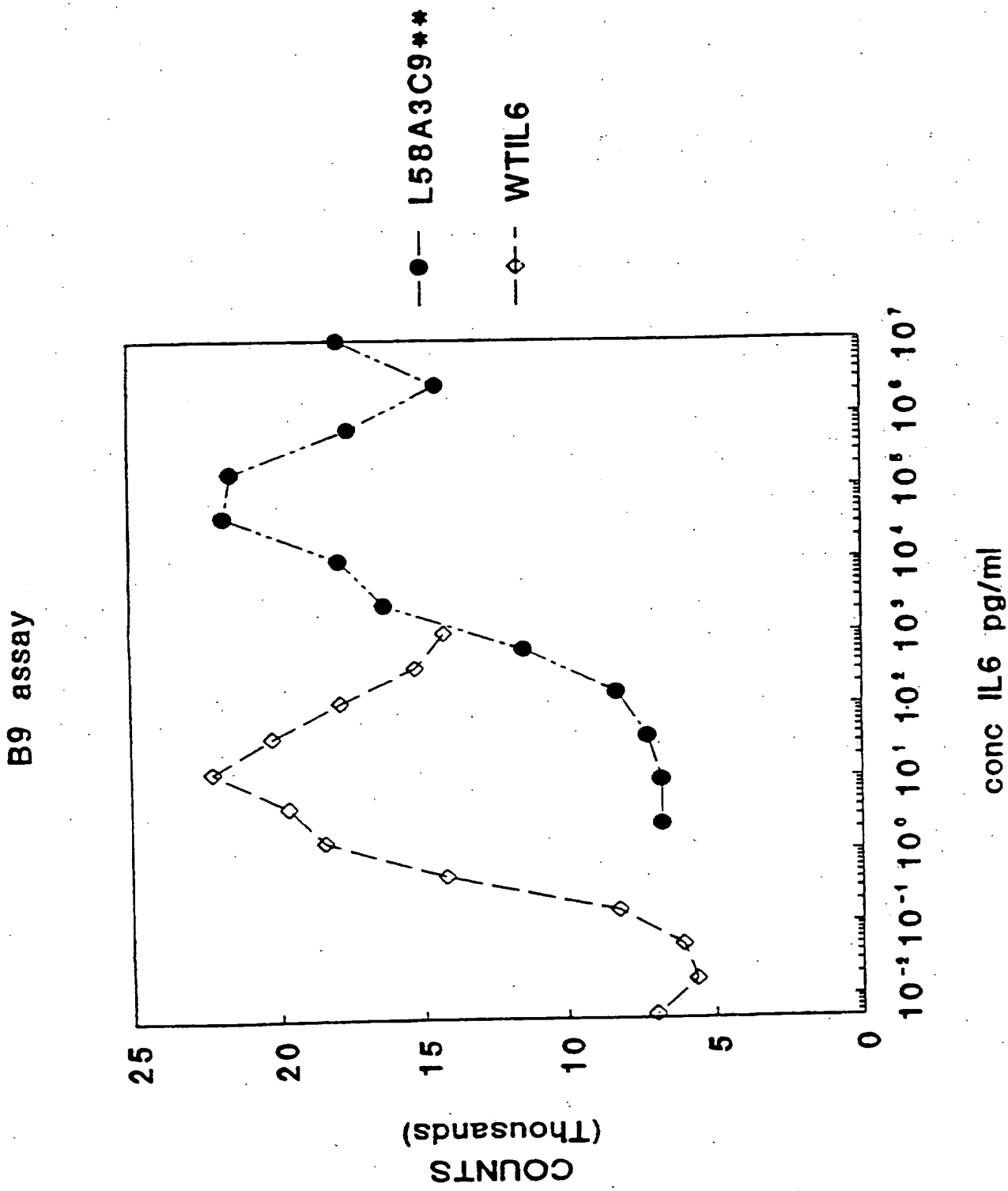
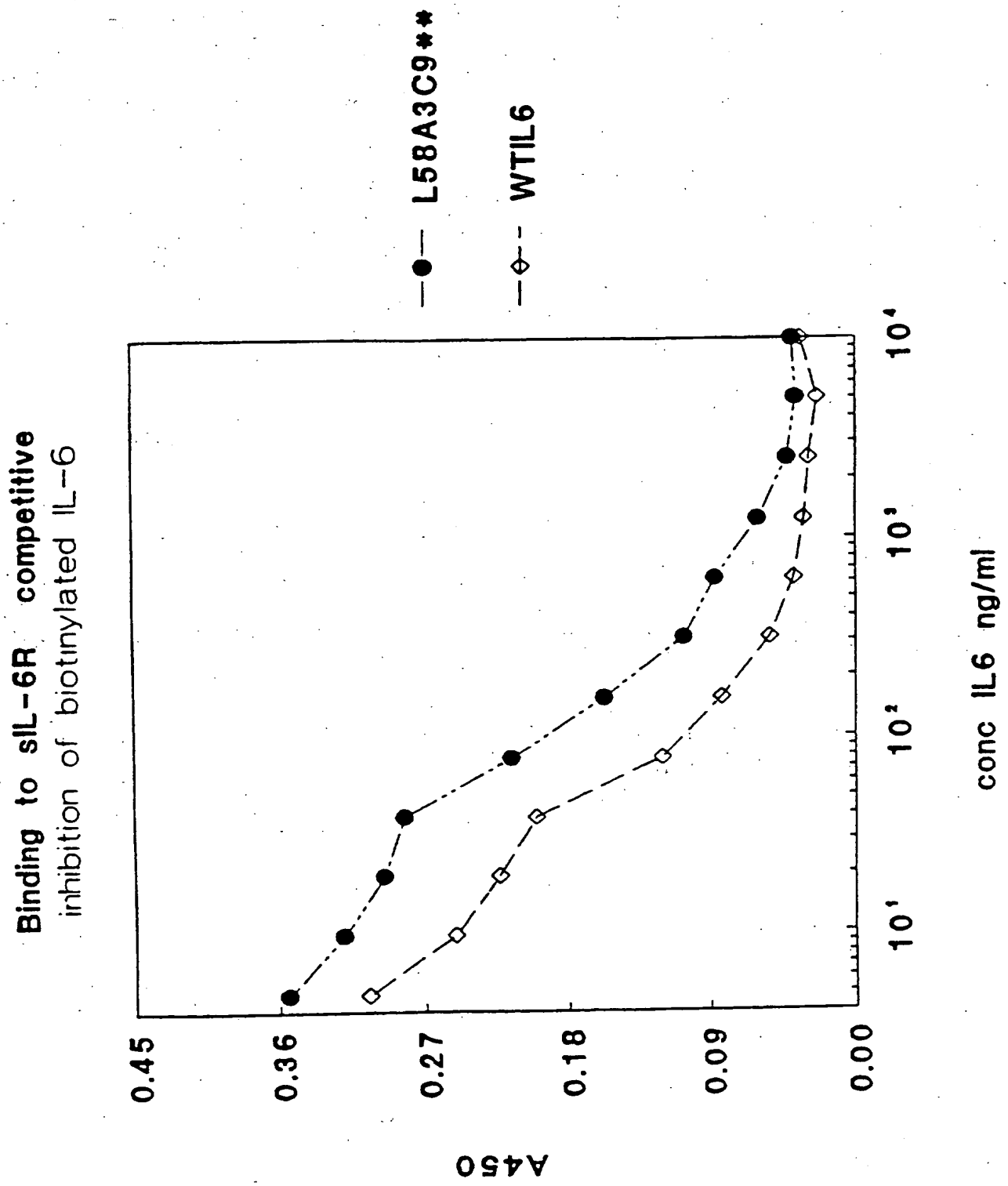

L58A3C9**
WTIL6
B9 assay
COUNTS
(Thousands)
25
20
15
10
5
0
10^{-2} 10^{-1} 10^{0} 10^{1} 10^{2} 10^{3} 10^{4} 10^{5} 10^{6} 10^{7}
conc IL6 pg/ml

Figure 10


Binding to sIL-6R competitive
inhibition of biotinylated IL-6
L58A3C9**
WTIL6
A450
0.45
0.36
0.27
0.18
0.09
0.00
10¹
10²
10³
10⁴
conc IL6 ng/ml

INTERNATIONAL SEARCH REPORT

Inter nal Application No
PCT/US 96/13981

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N15/24 C07K14/54 A61K38/20

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WO,A,94 09138 (CETUS ONCOLOGY CORP ;CENTRAL LAB OF THE NETHERLANDS (NL); BRAKENHO) 28 April 1994 see claims 1-24 ---	1-6,11
A	WO,A,94 11402 (ISTITUTO DI RICERCHE DI BIOLOG ;CILIBERTO GENNARO (IT); SAVINO ROC) 26 May 1994 see claims 1-9 ---	1-6,11
A	US,A,5 210 075 (SCHOLZ WOLFGANG ET AL) 11 May 1993 see example 5; table 2 ---	1-6,11

-/--

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
14 January 1997	29. 01. 97

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+ 31-70) 340-2040, Tx. 31 651 epo nl, Fax (+ 31-70) 340-3016	Gurdjian, D

Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

Interr nal Application No
PCT/US 96/13981

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	CYTOKINE, JUL 1995, 7 (5) P398-407, UNITED STATES, XP000615539 DE HON FD ET AL: "Functional distinction of two regions of human interleukin 6 important for signal transduction via gp130." see the whole document -----	1-6,11

3

Form PCT/ISA/210 (continuation of second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

ernational application No.
PCT/US 96/13981

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
Please see Further Information sheet enclosed.

2. [ ] Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. [ ] As all searchable claims could be searches without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International Application No. PCT/US 96/ 13981

FURTHER INFORMATION CONTINUED FROM PCT/ISA/210

Remark : Although claims 7-10 are directed to a method of treatment of (diagnostic method practised on) the human/animal body the search has been carried out and based on the alleged effects of compound/composition.

INTERNATIONAL SEARCH REPORT

information on patent family members

Inter ›nal Application No
PCT/US 96/13981

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
WO-A-9409138	28-04-94	AU-A-	5409294	09-05-94
		CA-A-	2147466	28-04-94
		EP-A-	0672144	20-09-95
WO-A-9411402	26-05-94	IT-B-	1263022	23-07-96
		CA-A-	2129824	26-05-94
		EP-A-	0667872	23-08-95
		JP-B-	2515491	10-07-96
		JP-T-	7501947	02-03-95
US-A-5210075	11-05-93	NONE		